The role of arginine in human wound healing

THE ROLE OF ARGININE IN HUMAN WOUND HEALING

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PROEFSCHRIFT

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Aan mijn ouders

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Chapter 1

Introduction

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Introduction

The skin is the largest organ of our body, it's surface expands up to 2 square meters in an adult person. Different functions are ascribed to the skin: it provides a physical barrier, which protect us against outside influences (temperature, ultra violet radiation, micro-organisms); the skin warns us if there are changes in temperature, senses pressure and pain. In addition, our skin regulates our internal temperature through perspiration and by vasodilatation. Next to these physiological functions skin also has an important role in social interactions. Man communicates through their skin e.g. skin shows how we feel inside (mental state), we touch others with our skin and visa versa. Moreover, we use our skin to express ourselves (make-up, tattoos, piercings etc.).

The skin has regenerative abilities, needed to restore any disruptions in order to protect us from outside influence. Adequate wound healing is essential for us plastic surgeons. Treatment of wounds has a long history that goes back to the ancient Egypt and Greece. The Ebers Papyrus, circa 1500 BC, detail the use of wound treatments (lint, animal grease, honey)[1]. Hippocrates described the importance of draining pus from a wound and Galen detailed the principle of primary or secondary healing[2,3]. The first research important for wound healing dates from the 19th century, when development of microbiology and cellular pathology occurred, by prominent physicians like Van Leeuwenhoek, Semmelweis, Lister and Virchow[4]. From then on we learned many things about normal wound healing physiology, which even led to therapeutical agents improving wound healing. However, the ultimate therapeutical agent still remains to be discovered. The complexity of wound healing is responsible for this. Normal wound healing is classically divided into three sequential phases: inflammation, proliferation and remodeling phase. During the inflammatory phase neutrophils, macrophages and lymphocytes clean the wound area from foreign material and prevent further bacterial colonization. Proliferation is characterized by multiple reparative cells, mainly fibroblasts. They form the structural proteins for a new matrix. In this phase endothelial and epithelial cell proliferation occur, leading to angiogenesis and epithelialization. During remodeling, the wound contracts to form a mature scar. The newly formed collagen is reorganized, resulting in increasing wound strength.

Different factors such as nutrition, radiation, ischemia, infection, diabetes, and medication influence wound healing. In this thesis we focused on nutrition and more specifically on **arginine**. The amino acid arginine and one of its metabolites nitric oxide (NO) have gathered broad attention in the last decades. This is related to the fact that arginine is not merely a component of proteins, but also participates in various metabolic processes, and is the direct precursor of NO. As such, arginine has been suggested to have important functions in pathophysiological events, such as sepsis, trauma and wound repair[5-9]. In most mammals, arginine is traditionally considered a conditionally essential amino acid[10-13], because during growth and metabolic stress the

endogenous production of arginine can become insufficient[14,15]. Normal daily intake of arginine is about 5-6 grams[16,17], whilst whole body arginine flux ranges between 15 and 20 grams per day[18-20]. Apart from being an essential component of proteins, arginine plays a key role in several other metabolic pathways[21,22]. It is a precursor in the synthesis of polyamines: putrescine, spermine and spermidine[5,23]. These compounds are important in the growth and differentiation, for example of intestinal mucosal cells[23]. Arginine is also a precursor for urea synthesis in the liver[24] as well as in the kidney[25,26], and as such plays an important role as a waste nitrogen carrier in the urea cycle. Besides, arginine is a precursor in the hepatic and renal synthesis of creatine[16,17,27], an important constituent of skeletal muscle[22]. Moreover, arginine appears to be converted by the enzyme arginine decarboxylase (EC 4.1.1.19) to agmatine, a metabolite that has been suggested to play a role in cell signaling and proliferation[22].

Via conversion by arginase, arginine is an indirect precursor for collagen formation through ornithine and proline, and as such involved in production of extracellular matrix molecules by fibroblasts[28], both important in wound healing. Arginine has also been suggested to have immunotrophic effects. It stimulates release of growth hormone and its peripheral mediator IGF-1, stimulates release of prolactin[21,23] and glucagon[16] and has the strongest insulinogenic effect of all amino acids[5]. Besides, both arginine and NO are important mediators in T-cell mediated immunity[29], cytokine induction[30] and macrophage mediated bacterial toxicity[31].

Arginine is the sole precursor of nitric oxide (NO) synthesis through the nitric oxide synthase (NOS) pathway (EC 1.14.13.39)[19,32]. Three isoforms of NOS exist: NOS1, NOS2 and NOS3. All isoforms appear to be present in non-stimulated conditions, including NOS2[33,34]. Microbacterial products and inflammatory cytokines can upregulate NOS2[35]. NO currently receives considerable attention in view of its widespread effects, especially in the cardiovascular system[36,37], where it functions as a signal molecule in vasodilatation and angiogenesis[38]. Through the same mechanisms, NO is involved in wound healing as it provides the wound area with sufficient oxygen and nutrition, which stimulates fibroblasts to synthesize collagen and keratinocytes to proliferate. Therefore NO takes an important part in building up repair-tissue in the wound.

In mice and rats with acute wounds, arginine was shown to be involved in wound healing via two metabolic pathways[15-18,39-44]. The first pathway is mainly active in the first three days and is catalyzed by NOS2, with citrulline and NO as end products. After a few days a shift to the second pathway occurs, catalyzed by arginase, which converts arginine into ornithine and urea. Ornithine is a precursor for collagen synthesis and cell growth. Schaffer and Lee[15-18,39,42-44] demonstrated consumption of arginine during acute wound healing. In their studies, arginine levels in wound fluid of mice were not detectable, whereas end products of arginine metabolism were increased, thus suggesting local use of arginine in wounds. One of the products of arginine is ornithine, in itself a precursor for collagen synthesis. Studies with NOS2-knock-out mice supplied

with dietary ornithine show increased wound breaking strength and collagen deposition.

In experimental studies with mice, Witte and Schaffer demonstrated the essential role of **NO in wound healing** by showing that NOS2-knock-out mice had delayed wound healing[8] and NO inhibitors diminished wound collagen formation[28]. In tendon healing, Murrell et al.[45] showed an increase in NOS activity during healing. Furthermore, NOS2 inhibition resulted in decreased tendon healing. Similar results were found concerning NO and fracture healing[46]. In experiments with NOS3-deficient mice, wound closure was delayed, tensile strength decreased and endothelial cell sprouting reduced[38]. This upregulation of the enzymes involved in the NO-pathway suggest an increased need for arginine in wound healing, as arginine is the sole precursor for NO.

Subsequently the hypothesis that **arginine supplementation** might be beneficial for wound healing was evaluated in several studies. In rats, 300 mg/kg of arginine per day enhanced wound tensile strength, hydroxyproline concentrations and collagen accumulation. In addition, it decreased neutrophil counts and adhesivity[47]. In NOS2 knock-out mice, NOS2 was shown to be a mediator in the effects of arginine supplementation on wound breaking strength and hydroxyproline content[48]. Arbbs et al. studied the effects of high arginine levels on early integration of biocompatible mesh grafts in rat abdominal wall. They found enhanced cell accumulation, formation of new vessels, fibroblast proliferation and type III collagen deposition in the arginine-supplementation group[49]. In an experimental model for ileitis, ulcer healing was enhanced in rats supplied with diets containing 5% arginine[50]. Healing of colonic anastomosis in rats on postoperative day 6 was enhanced when rats were fed a peri-operatively arginine enriched diet[51]. Arginine supplementation improved wound immune cell function in mice, demonstrated by decreased pro-inflammatory IL-1β, IL-6 and increased anti-inflammatory IL-10 [52]. Arginine supplementation also affected angiogenesis. Angele et al. demonstrated increased microcirculation after arginine supplementation in mice with depressed skin and muscle blood flow after hemorrhage[52]. Arginine supplementation in a rabbit ischemic hind limb stimulated angiogenesis, as shown by marked increase in capillary density and enhanced collateral vessel development[53]. NOS3 was essential for angiogenesis in ischemic tissues in this model.

Diabetes is characterized by a NO deficient state at the wound site. In diabetic rats, arginine supplementation increased wound breaking strength. At the same time, plasma arginine and NO_x levels were increased, while wound fluid levels of arginine and ornithine remained unaffected[54]. This suggests that the exogenously provided arginine is metabolized to NO by NOS2. In accordance, supplementing the NO donor molsidomine to these rats increased wound breaking strength, hydroxyproline content, urinary NO excretion and MMP-2 activity. It also decreased collagen I and III gene expression and arginase activity[55].

Only few **human studies** investigated the effect of arginine as *single* pharmaco-nutrient on wound healing. Kirk et al. supplied thirty healthy elderly humans with

17 grams of arginine per day and found improved serum IGF-1, enhanced hydroxyproline and total protein content deposition in subcutaneously placed catheters[56]. Supplementation however, did not shorten time to complete epithelialization of skin defects of 2 cm². Barbul et al.[5] supplied arginine to 36 young healthy humans, divided in three groups, which received either 17 or 25 grams free arginine or placebo for 2 weeks. Both arginine-supplemented groups displayed enhanced hydroxyproline accumulation in subcutaneously placed catheters. Besides, lymphocyte immune reactivity was enhanced. So far, no human studies on the effect of arginine on angiogenesis in wound healing have been published. Moreover, the performed studies address experimental wounds, not relevant in clinical practice.

Aim of the thesis

Arginine supplementation has found its way into clinical practice. However, conclusions regarding arginine supplementation derive mainly from studies using commercially available nutritional supplements containing, besides arginine, other nutrients such as nucleotides and ω-3 fatty acids (e.g. Impact®, Novartis, Minneapolis, Minnesota, USA or Immune-Aid®, McGaw, Irvine, California, USA). In general, two major problems exist with this kind of studies: 1) A mixture of nutrients is used: from a scientific point of view, this complicates interpretation of the results, since it is impossible to distil to what nutrient the effects should be contributed; besides, ingredients could interact antagonistically. 2) Control groups are often not present or are not supplied with isonitrogenous and/or isocaloric amounts: this makes it difficult to conclude whether treatments should be attributed to the specific pharmaconutrients or simply to the addition of amino acids and/or calories in general. The overall objective of this thesis is to contribute to a further understanding of:

1. the role of arginine in healing of surgical (acute) wounds (chapter 3)
2. the role of arginine in chronic wounds (chapter 4)
3. arginine metabolism in normal human skin (chapter 2)
4. the effect of arginine, as a monotherapy, on wound healing in a randomized controlled trial (chapter 5 and 6)

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Chapter 2

Arginine metabolism in human skin; using free vascularized skin flaps as a model

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In submission

Abstract

Background
The skin is the body's largest organ; it protects the body against fluid loss and invasion of micro-organisms. When it becomes damaged, arginine supplementation improves its healing in rodents. This is attributed to the local conversion of arginine to its metabolites nitric oxide (NO) and ornithine. However, amino acid metabolism within the skin and definite mechanisms by which arginine supplementation may improve wound healing remain unknown. Aim of this study was to investigate the physiology of amino acid metabolism in human skin.

Methods
Eleven patients undergoing reconstructive surgery using a free vascularized skin flap received a primed continuous intravenous infusion of $^{15}N_2$-arginine. Arterial and venous blood was sampled and arteriovenous gradients of unlabeled and labeled amino acids were measured to assess skin uptake, conversion, and release of arginine, citrulline, ornithine and other amino acids.

Results
Normal human skin showed significant net uptake of $^{15}N_2$-arginine . This was accompanied by net release of citrulline and ornithine. No net release of ^{15}N-labelled citrulline or urea could be demonstrated. Net release of essential and other protein bound amino acids reflected protein breakdown.

Conclusion
This is the first study of skin amino acid metabolism in humans. Arginine was taken up by the 'normal' skin, probably to serve as precursor for both NO and ornithine production. These data support the view that arginine metabolism is important in skin physiology and wound healing and provide rationale for further research concerning the role of arginine supplementation in acute and chronic wounds.

Introduction

The skin is the largest organ of the human body; it protects the body against fluid loss and invasion of micro-organisms. Disruptions of this barrier are common after trauma, surgical interventions and burns. The response is immediate repair of the skin's\integrity by wound healing. Knowledge of skin nitrogen metabolism is important to understand this complex process of wound healing. Numerous mediators play a role in wound healing. The last decade, the amino acid arginine has received a lot of attention. In normal healthy conditions the human body can generate a substantial amount of arginine endogenously[1]. However during stress situations (sepsis, trauma, cancer), endogenous arginine production may not be sufficient to match the arginine requirements[2-5]. Arginine is therefore considered a conditional essential amino acid. Arginine can be degraded enzymatically by the enzymes arginase or nitric oxide synthase. The enzyme arginase (ASE) converts arginine to urea and ornithine[6]. Ornithine can be metabolized to polyamines, that play an important role in cell growth and proliferation. Alternatively, ornithine can be converted to proline, a collagen precursor, which is essential for wound healing.Two isotypes of ASE exist; ASE-1 is liver arginase, part of urea cycle. ASE-2 is a mitochondrial enzyme in many tissues outside the liver. Nitric oxide synthase (NOS) converts arginine to nitric oxide (NO) and citrulline[7,8]. The short-living molecule NO is involved in regulating many processes like neurotransmission, angiogenesis and immune response. Previous studies have shown the importance of arginine in experimental wound healing as supplementation with arginine improves wound healing whereas arginine-free diets impair wound healing[9-16]. Changes in plasma and wound fluid concentrations are used as markers for activities of specific arginine metabolic enzymes. A decrease in wound fluid levels of arginine and an increase in ornithine, citrulline and NOx are observed, suggesting arginine conversion by NOS and arginase[17-20]. Human studies have shown that diets enriched with arginine stimulate the immune system and wound healing (using hydroxyproline content in granulation tissue as a marker)[16,21-30]. However, potential mechanisms by which arginine supplementation may affect wound healing remain unclear. The aim of this study was to investigate arginine metabolism in normal human skin. The studies were conducted in patients undergoing free vascularised skin flap transplantation. This enabled us to measure arteriovenous concentration gradients of labelled and unlabelled amino acids across an isolated segment of the human skin.

Materials and methods

Patients

A total of 11 patients (all female, median (range) age 43 (39-53), admitted for elective plastic surgery, participated in this study conducted at the department of Plastic

Surgery, University Hospital Maastricht, the Netherlands. Median weight and BMI were 80 (60-87)kg and 29.6 (24.0-35.6) respectively. All patients underwent reconstructive surgery using a free vascularized DIEP flap (Deep Inferior Epigastric artery Perforator). This is a perforator-based skin flap, consisting of both skin and subcutaneous fat[31]. Table 2.1 shows the patients characteristics.

Patients known with active cancer, liver disease, inborn errors of metabolism, insulin-dependent diabetes or use of steroids were excluded from the study. On the day of admission routine blood tests were performed. Patients were fully informed about the purpose of the study and the potential risks involved. The study was approved by the Medical Ethical committee of the University Hospital Maastricht and all patients gave written informed consent. Oral intake, except for water, was ceased at 12 am on the day of admission and all patients were transported to the operation theatre at approximately 7:30 am the next day. All surgical procedures were performed under general anesthesia. Skin flaps were dissected using standard operative techniques and performed by the same plastic surgeon.

Table 2.1 Patient characteristics.

	DIEP Median/range
Male/Female	0/11[A]
Active cancer	0[A]
Age (years)	43(39-53)
Weight (kg)	80.0 (60.0-87.0)
BMI (Kg/cm2)	29.6 (24.0-35.6)

Tracer infusion and blood sampling

Surgery was performed following an overnight fast, anaesthetic management was according to institutional routines and included placement of two peripheral venous catheters and an arterial line. Anaesthesia was maintained using isoflurane and propofol. After induction of anaesthesia, an additional peripheral venous catheter was placed in an antecubital vein for tracer infusion. This catheter was kept patent with normal saline until the start of the tracer infusion. A baseline blood sample was drawn from the arterial line, followed by the start of a primed continuous intravenous tracer infusion. Patients received priming doses in 45 ml normal saline solution of L-[guanidino-$^{15}N_2$]arginine followed by constant intravenous infusion of L-[guanidino-$^{15}N_2$]arginine (3.51 µmol/kg/h), for two hours (Cambridge Isotope Labs, Andover, MA, USA). Infusates were prepared from sterile, pyrogen-free powders. Subsequently, blood samples were collected from an arterial line or peripheral venous line at 60 min, 90 min, 105 min and 120 min after infusion to determine steady state conditions. At t=120 min, when the flaps were dissected, arterial and venous blood samples were taken from the vascularized skin flaps. First the draining vein was cannulated and 10 ml

of blood was collected, no forced suction was used during blood collection, to prevent hemolysis. The time needed to collect this amount of blood was recorded to calculate blood flow through the flap, expressed as ml/min. Simultaneously, an arterial blood sample was collected from a central arterial line or from the supplying artery of the skin flap through cannulation.

Sample processing and analysis

Blood was collected in pre-chilled heparinized vacuum tubes (BD Vacutainer, Franklin Lakes, NJ), placed on ice, and processed as described before[32]. Within one hour, blood was centrifuged (10 min, 4000 rpm, 4°C) and 500 µl of plasma was added to 80 mg dry sulphosalicylic acid (Across Inc., Geel, Belgium) to precipitate plasma proteins. After vortex mixing deproteinized plasma samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. The skin flap was weighted and the surface measured.
Amino acid concentrations in deproteinized samples and infusates were measured using high performance liquid chromatography as described elsewhere[33]. Urea concentrations were determined by the routine clinical chemistry lab of our hospital. Arginine, citrulline and urea enrichments were measured by liquid chromatography-mass spectrometry[34].

Calculations of amino acid kinetics

Isotopic enrichment was expressed as tracer-to-tracee ratio (TTR, %), taking into account the contribution of overlapping isotopomer distributions of the tracee and tracers with lower masses to the measured TTR as described by Vogt et al.[35]. Within 30 minutes isotopic steady state was achieved (data not shown).

Since blood flow data yielded by the applied methods appeared to be unreliable transorgan concentration gradients rather than transorgan fluxes were calculated. The amino acid net balance (NB) was calculated as the difference between arterial ([A]) and venous ([V]) amino acid plasma concentrations:

$$NB = [V] - [A]$$

Accordingly, a positive balance indicates organ specific net release whereas a negative balance indicates net uptake.

The net balance of labelled amino acids (tracer net balances, tNB) was calculated by multiplying arterial and venous amino acid concentrations with amino acid arterial and venous enrichments (TTR_A, TTR_V):

$$tNB = ([TTR_A \times [A])-(TTR_V \times [V])$$

Absolute arginine uptake by the skin flaps was calculated from tracer net balance and $^{15}N_2$ arginine enrichment of the venous plasma sample:

absolute uptake = tNB/TTR_V

Statistics

Because of the small sample size non-parametric analyses were selected. Arteriovenous gradients and tracer net balances were tested versus a theoretical mean of zero using a Wilcoxon signed rank test. Correlations between arteriovenous concentration differences of arginine, citrulline and other amino acids were calculated with the use of the Spearman's test. Results are expressed as median and interquartile ranges (IQR). All statistical calculations were performed using Prism 4.0 for Windows (GraphPad Software Inc. San Diego, CA). A p-value <0.05 was considered to indicate statistical significance.

Results

Experimental studies were completed as scheduled in all 11 patients and no protocol violations occurred. Surgical procedures were successful in all cases, particularly the perfusion of the flaps was maintained intact throughout the infusion of $^{15}N_2$ arginine. Isotopic steady state was achieved within 30 minutes in all patients and maintained throughout the remainder of the experiments (data not shown). Median (IQR) arterial $^{15}N_2$ arginine enrichment was 6.0 (5.0-8.1) %.

Arginine uptake and metabolism

The DIEP flaps significantly extracted $^{15}N_2$ arginine from the circulation (Figure 2.1). The arterial arginine concentration was 90 (58-99) µmol/l. The median (interquartile range) absolute arginine uptake by the DIEP flaps was 19.0 (7.2-33.2) µmol/l (p=0.007). From these numbers, a fractional arginine extraction of 13.5 (2.4-22.3)% could be calculated. There was however no correlation between arterial arginine supply and arginine uptake in the skin flaps (Figure 2.2).

The human skin flaps significantly released ornithine and citrulline, evidenced by arteriovenous concentration gradients that both significantly differed from zero 8.0 (5.8-8.8) µmol/l and 1.5 (0.5-3.4) µmol/l respectively (both p<0.005) (Figure 2.1).

Somewhat conflictingly, no significant release of ^{15}N labeled products of arginine metabolism i.e. ^{15}N urea, representing arginase activity or ^{15}N citrulline, representing NOS activity could be observed in the present study. The arteriovenous concentration gradient of nitrite was -0.07 (-0.9 – 0.04) µmol/l, however this gradient failed to reach statistical significance when compared to a theoretical median of zero (p=0.08).

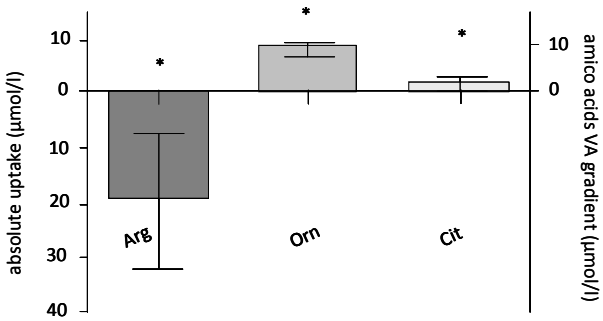

Figure 2.1 Median (interquartile range) absolute uptake of 15N2-arginine is expressed (left Y-axis) and median (interquartile range) amino acid net balance of citrulline and ornithine (right Y-axis), both expressed in µmol/l, * significant difference from zero p<0.05.

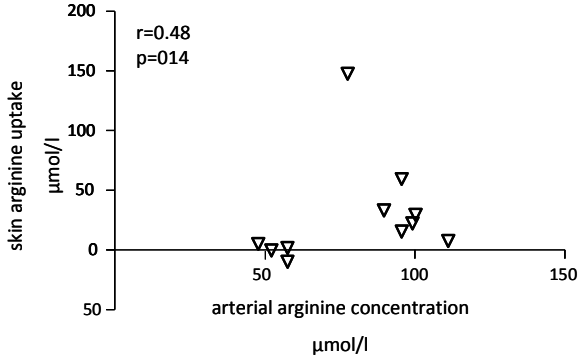

Figure 2.2 Correlation between arterial arginine concentration and uptake of arginine by the human skin. There was no significant correlation, which suggests that arginine uptake by the human skin is not dependent of on its influx. Spearman's test for non-parametric correlations (Spearman's rho =0.48, p=0.14).

Metabolism of other amino acids

A net release of virtually all amino acids from the skin flaps was observed. Most importantly, net release of essential amino acids was found, which must be ascribed to net protein breakdown. There was net uptake of glutamate. The net arteriovenous gradient of urea was approximately 2% of the arterial concentration and therefore too small to assess reliably. Table 2.2 summarizes the arterial concentrations and arteriovenous gradients of amino acids and other nitrogenous compounds across the flaps.

Table 2.2 Arterial [A] concentrations and arteriovenous concentration gradients across human skin flaps [A-V] of amino acids and other nitrogenous compounds, expressed as median [range]. Positive gradients indicate uptake, negative gradients release. * arteriovenous gradient significantly different from zero, p<0.05.

Amino acid	[A](μM)	[A]-[V] (μM)
Urea	3600 [1800-5500]	2000 [-1000-800]
NO2	1.22 [1.1-1.38]	-0.07 [-0.95-0.04]
ARG	89 [48-111]	-11 [(-25)-4]*
GLU	67 (38-102)	14 (5-39)*
ASN	56 (34-71)	-11 [(-19)–(-3)]*
SER	122 [89-198]	-18 [(-32)-(-10)]*
GLN	1028 [771-1295]	-175[(-284)-(-100)]*
HIS	83 [69-156]	-31 [(-42)-(-19)]*
GLY	312 [149-556]	-41 [(-88)-(-13)]*
THR	169 [92-224]	-22[(-36)-(-4)*
ALA	417 [214-718]	-85 [(-273)-(-17)]*
TAU	47 [34-75]	-51 [(-86)-(-23)]*
aAB	25 [12-37]	-2 [(-4)-1]*
TYR	47 [28-76]	-8 [(-16)-(-3)]*
VAL	197 [103-245]	-17 [(-23)-7]*
MET	27 [17-35]	-5 [(-8)-(-1)]*
ILE	57 [17-84]	-4 [(-9)-3)*
PHE	57 [35-77]	-8 [(-18)-(-1)]*
TRP	23 [16-29]	-2 [(-7)-1]*
LEU	123 [50-185]	-10 [(-26)-5]*
LYS	191 [113-241]	-31 [(-52)-(-11)*

Discussion

We have studied arginine metabolism of healthy human skin by using transorgan balances of skin flaps during breast reconstruction procedures. The use of stable isotope techniques enabled us to calculate production and consumption. By labelling arginine with two ^{15}N isotopes at its guanido group its uptake by the skin tissues and its enzymatic conversion to citrulline (and NO) by NOS and urea (and ornithine) by arginase could be assessed. We found significant uptake of $^{15}N_2$arginine, in addition a net release of ornithine and citrulline was observed. Median net arginine uptake was 15.5 μmol/l, with a fractional extraction of 13.5%. Ornithine is formed from arginine by ASE, also yielding urea. While citrulline is a product of NOS. Both ornithine and citrulline are no constituent building blocks of protein and therefore their net release by the human skin flaps cannot be ascribed to protein breakdown and must therefore uniquely reflect de novo synthesis by enzymatic conversion of arginine. Degradation of $^{15}N_2$ arginine would result in production of $^{15}N_2$ urea and unlabeled ornithine. In addition, NOS would give rise to ^{15}Ncitrulline and ^{15}NO. We could not measure such enzymatic conversion this was probably due to the arteriovenous concentration

differences of labelled citrulline and urea, being too small to be detected by the applied methods.

Furthermore, net amino acid fluxes show that glutamate is taken up by the skin (table 2) and essential amino acids like phenylalanine are released. The latter indicates net protein degradation in the skin flaps. Previous experimental studies in a rabbit skin model show similar findings[36,37]. Although tracer data clearly show arginine tracer uptake by the skin, amino acid balance shows net arginine release. In line with the general net release of amino acids this finding can be ascribed to net protein breakdown in these post-absorptive patients. The unique uptake of the amino acid glutamate is remarkable. Glutamate is a precursor for glutamine, which has several important functions: it is a gluconeogenic precursor, a vehicle for interorgan carbon/nitrogen transport, metabolic substrate for cells with high turnover rate (immune cells, enterocytes, renal cells). In addition, it is a precursor for purines/pyrimidines needed for DNA-synthesis and gluthathione an anti-oxidant. In times of catabolic stress glutamine is released into plasma, specifically by muscle, but also by adipose tissue[38-40]. As precursor for proline, glutamate might have a role in collagen formation, essential for wound healing[41]. Glutamate uptake by the legs has been described in several experimental settings, including in multi-catheterized humans[37]. This has invariably been ascribed to conversion to glutamine by skeletal muscle glutamine synthetase-activity. Our data suggest that skin, next to skeletal muscle, may be involved in the conversion of glutamate to glutamine in the extremities. Dermis seems to have an efficient reuse of amino acids, resulting in net protein balance of zero[36], therefore layer of subcutaneous tissue incorporated in our skin flaps may be responsible for this finding. Which would be in line with in vitro studies showing glutamine metabolism in adipocytes[39]. Next to muscle, fat may be an important producer of glutamine in times of systemic distress[38,42]. Whether this conversion is needed for systemic or local processes, or ammonia detoxification remains unknown.

Most studies implicating the importance of arginine for skin healing, have been performed in rodents. In these studies arginine consumption is shown in wounds, and in addition they show that arginine supplementation improves nitric oxide-mediated wound healing[5,17,20,43,44].

In line with experimental studies we previously observed an up-regulation of arginine metabolizing enzymes in human wounds compared to uninjured skin, suggesting arginine metabolism[44]. However, we could not show a beneficial effect of arginine supplementation on human wound healing in our RCT's[45,46]. An explanation might lie in the present study showing no correlation between uptake of arginine by the skin flaps and their arterial arginine supply, indicating that increasing arginine influx does not lead to increased uptake, and therefore suggesting that arginine metabolism in the skin is not a substrate-driven process.

The inaccessibility of afferent and efferent blood vessels of skin was a drawback to measure arteriovenous balances across skin. The rabbit ear model, as introduced by

Zhang et al.[36] was the first animal model in which it was possible to measure flow and sample arteriovenous balances across an isolated peace of skin. This model shows net protein breakdown in normal rabbit skin. However, arginine metabolism was not measured. The technique of microsurgical free vascularized skin transplantations used by plastic surgeons since 1975, allowed us to sample veins and arteries separately. For the first time we used this model successfully in men, making it an unique model for future research.

In conclusion, this is the first study of transorgan measurements of arginine metabolism in human skin. The data support our hypothesis of arginine consumption to produce NO and ornithine in human skin. These findings provide rationale for further research after the role of arginine metabolism in wound healing.

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Chapter 3

Role of arginine in superficial wound healing in man

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Abstract

Backgroud
Arginine supplementation has been identified as advantageous in experimental wound healing. However, the mechanisms underlying this beneficial effect in tissue repair remain unresolved. Animal studies suggest that the beneficial role of arginine supplementation is mediated, at least in part through NO. The latter component mediates processes involved in tissue repair, including angiogenesis, epithelialization and collagen formation.

Methods
This prospective study is performed to investigate arginine metabolism in acute surgical wounds in man. Expression of enzymes, known to be involved in arginine metabolism were studied in donorsites of skin grafts of 10 hospitalized patients undergoing skin transplantation. Plasma and wound fluid levels of arginine metabolites (ornithine, citrulline, nitrate and nitrite=NOx) were measured using High Performance Liquid Chromatography. Expression of iNOS, eNOS, arginase-1 and arginase-2 were studied by immunohistochemistry in paraffin sections of skin tissue. Arginase-1 concentration was measured in plasma and wound fluid using ELISA. Arginase-2 was determined using Western Blot analysis.

Results
We observed increased levels of citrulline, ornithine, NOx and arginase-1 in wound fluid when compared with plasma. Arginase-2 was expressed in both plasma and wound fluid and seemed higher in plasma. iNOS was expressed by neutrophils, macrophages, fibroblasts, keratinocytes and endothelial cells upon wounding, whereas eNOS reactivity was observed in endothelial cells and fibroblasts. Arginase-1 was expressed in neutrophils post-wounding, while arginase-2 staining was observed in endothelial cells, keratinocytes, fibroblasts, macrophages and neutrophils.

Conclusion
For the first time, human data support previous animal studies suggesting arginine metabolism for an NO- as well as arginase-mediated reparation of injured skin.

Introduction

Wound healing represents a complex process, initiated to restore tissue damage. The amino acid arginine has been identified as an important mediator in this process[1]. Arginine is the sole precursor of nitric oxide (NO), a signal molecule, among others, involved in immune responses, angiogenesis, epithelialisation and formation of granulation tissue, all essential aspects accompanying wound healing[2].

Nitric Oxide Synthase (NOS) converts arginine to NO and citrulline. Three isoforms of NOS exist: Neuronal NOS (NOS 1) and endothelial NOS (NOS3) are constitutively expressed by neuronal and endothelial cells respectively. Inducible NOS (NOS 2), is expressed in response to inflammatory cytokines and endotoxins, such as seen during wound repair. The beneficial effects of arginine supplementation on wound healing have been attributed to enhanced synthesis of NO by NOS[3-6]. Former studies with rodents showed that arginine-free diets impair wound healing, with decreased breaking strengths of incisions and collagen deposition in granulation tissue)[7,8], while supplementation of arginine increased hydroxyproline concentration, a marker of collagen synthesis[9-12]. In addition, to its role as precursor of NO, arginine can be metabolized by arginase[13-17]. Two different isoforms of this enzyme have been identified: arginase-1 (ARG1) and arginase-2 (ARG2). The liver type, ARG1, is the cytosolic isoform. ARG2 is the mitochondrial isoform, located in kidney, prostate, small intestine and the breast. Arginase catalyses the conversion of arginine to ornithine and urea. Ornithine is an essential precursor for collagen and polyamines synthesis[18], both required for wound healing processes[19-25]. Moreover, arginase seems to influence the immune response, another important contributor involved in tissue repair. Activated macrophages and neutrophils (PMN's) show increased NO-production for their anti-bacterial function. This NO-production is subsequently down-regulated by arginase through substrate competition[26-28].

Although arginine supplementation is considered to promote the wound healing process, little is known of its metabolism during normal human wound healing. This study examines the role of arginine during uncompromised human wound healing. To this end, levels of arginine, its metabolites and arginase were measured in wound fluid and plasma of superficial surgical wounds in man. In addition, we evaluated the immunohistochemical distribution of the enzymes arginase, iNOS and eNOS in these wounds on paraffin-embedded tissue sections.

Materials and methods

A prospective study was performed in ten hospitalised healthy adults undergoing skin transplantation as part of reconstructive surgery at the Department of Plastic Surgery in University Hospital Maastricht. Details of their characteristics are given in Table 3.1.

Exclusion criteria were: age younger than 18 or older than 70 years, kidney or liver failure, pregnancy, use of steroids or diabetes mellitus.
Patients underwent screening evaluation, including medical history and physical examination. Nutritional assessment was obtained measuring body weight and length and expressed as Body Mass Index (= weight / lenght²). Age and sex of the patients were noted. The Maastricht Hospital Medical Ethical Board approved the investigation protocol. Each patient signed informed consent.

Wound protocol

For the purpose of this study we used the donorsite of a skin graft as a model for an acute surgical wound. Under aseptic conditions and general anaesthesia skin grafts were obtained by using an electric dermatome (Aesculaap®) with a thickness of 0.3 millimeters. Biopsies of normal skin obtained on day zero (=day of operation) were used as controls. To collect wound fluid donorsites were covered with a layer of Gordasoft®, then a polyvinylalcohol (PVA) sponge was applied to the wound (Coldex®) and on top of that a transparent dressing (Tegaderm®), as previously described[29].

Study protocol

The initial protocol was to sample on day 2, 5 and 10 after surgery. However, on day 10 wound fluid samples could only be harvested in three patients, as in the other patients no wound fluid was present in the sponge. Twenty-four hours before sampling, the dressing was changed by removing the transparent dressing and the PVA-sponge and reapplying a new sponge and transparent dressing. On the day of sampling, biopsies were taken under local anesthesia (1% lidocaine), using a 3 millimeters punch biopsy from the central part of the wound. The sponge was removed, and immediately stored on ice. Simultaneously a venous blood sample was drawn from a major vein in the cubital fossa and also put on ice. After sampling a new dressing was applied to the wound.

Sample collection, processing and analysis

Blood was centrifuged at 4°C for 10 minutes (4000 rpm) within one hour after sampling. After centrifugation, 500 µl of plasma was deproteinized using 20 mg dry sulphosalicylic acid (SSA), vortexed and frozen in liquid nitrogen. Samples were stored at -80°C until analysis. To obtain wound fluid from acute wounds, sponges were centrifuged at 4°C for 10 min (11.000 rpm). After centrifugation, 500 µl of wound fluid was treated similar to the plasma.

Amino acid concentrations

Plasma and wound fluid amino acids were determined using a fully automated High Performance Liquid Chromatography system (HPLC) as described previously. Nitrate / nitrite concentrations in plasma and wound fluid were also determined using HPLC[6].

Nitrate / nitrite (NOx) concentrations

To study production of NO by NOS we measured nitrite and nitrate concentrations. The sum of both nitrite and nitrate (NOx) is used as an indirect indicator of NO production as described in several studies[6,14,30]. NOx concentrations in plasma and wound fluid were determined using HPLC[6,31].

Elisa for arginase-1

Plasma and wound fluid arginase-1 levels were measured using sandwich ELISA[32]. Standard, primary and secondary antibodies were kindly provided by HBT (Uden, The Netherlands). In short, a 96-wells plates were coated overnight at 4°C with 100 µl of the purified anti-human liver-type arginase monoclonal IgG (first antibody; Mo6G3, 5 mg/l carbonate buffer, pH 9.5). Plasma samples were prediluted 1:2 in dilution buffer (1x PBS-0.1% BSA) and wound fluid samples 1:400. Samples were added to the plate. Bound arginase was detected with a second biotinylated antibody (Mo9C5 in dilution buffer 1:200), followed by peroxidase-conjugated streptavidin and TMB.

Western blot arginase-2

Plasma or wound exudates were centrifuged at 10.000 rpm for 5 min, supernatants were collected and total protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL)
Aliquots with equal amounts of protein were heated at 100°C for 5 min in Laemmli buffer with ß-mercaptoethanol, separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). After incubation with primary antibody, 1 µg/ml anti-human arginase-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, USA.) and washing, membranes were incubated with a horseradish peroxidase–conjugated secondary anti mouse IgG. Positive bands were detected using the chemiluminescent substrate Supersignal West Pico (Pierce Chemical Co) and transferred onto an X-ray film.

Immunohistochemical staining

The cellular distribution of arginine-metabolising enzymes was studied in man during the process of wound healing. Sequential biopsies were collected in a period of ten days post-wounding. Skin tissue samples were stained with arginase-1, arginase-2, iNOS and eNOS and compared them to normal skin. For immunophenotyping a

standard staining with haematoxylin and eosin (H&E), alpha-smooth muscle actin (ASMA), CD31 and CD68 was performed (data not shown).
Biopsies were fixed in 4% buffered formaldehyde, processed by routine histological procedures and embedded in paraffin. Four micrometer sections were subsequently obtained from each paraffin block. Specimens were initially stained with H&E. Parallel sections were immunohistochemically stained for ASMA (smooth muscle cell staining, Dako, monoclonal mouse-anti-human, 1:500), CD31 (endothelial cell staining, Dako, monoclonal mouse-anti-human, 1:50, pre-treatment TRIS/EDTA/pH 8.0, blocking 5% BSA) and CD68 (macrophage staining, DAKO, monoclonal antibody, pre-treatment pepsin 1:100, no blocking). Subsequently sections were stained using polyclonal rabbit anti-human-iNOS antibody (Zymed Laboratories Inc. South San Francisco, USA), polyclonal goat anti-human eNOS antibody (R&D systems, Las Vegas, USA), rabbit-anti-arginase-1 (Sigma-Aldrich, St. Louis, USA)
and rabbit anti-human type-2 Arginase (kindly provided by Tomomi Gotoh, Kumamoto University, Japan). Briefly, slides were dewaxed and rehydrated in decreasing concentrations of alcohol. For arginase-1, slides were pre-heated in citrate buffer. Sections were then incubated for 30 min with iNOS, eNOS, arginase-1 and arginase 2 antibodies (resp. diluted 1:50, 1:10, 1:750, and 1:500 in 1% BSA/TBS/0.1% Tween-20 solution). Then slides were treated with biotinylated anti-rabbit (diluted 1:200, Dako) or biotinylated anti-goat (diluted 1:400, Dako) and treated with avidin-biotin-peroxidase-complex (Dako). Positive staining was visualized by applying alkaline phosphatase (Vector Laboratories, Inc. Burlingame) and slides were counter-stained with Mayer's haematoxylin. Negative controls were treated in the same manner but with the omission of the primary antibodies. The slides were independently reviewed in a blinded fashion by two observers, by light microscope. For determination of the wound score, slides were rated semi-quantitatively on a score of 0 to 4 for cells with cytoplasmic staining. 0 (negative staining); 1(0-25% of the cells stained positive); 2 (26-50% of the cells stained positive), 3(51-75% of the cells stained positive) and 4 (76-100% of the cells stained positive).

Statistical analysis

Data are expressed as mean ± SEM. Amino acid concentrations were expressed as μM. Differences of plasma and wound fluid parameters were analyzed using ANOVA repeat measurements tests from SPSS, this statistical package for social sciences (SPSS) software was used for statistical analysis. In all cases, $p<0.05$ was considered statistically significant.

Results

Patient characteristics

Patient characteristics are shown in Table 3.1. Eight males and two females entered the study, mean age was 47 years. Patients were in a general good condition. Mean body mass index was 26.5 and none of the patients had suffered from weight loss >10% of bodyweight prior to our study (data not shown). Patients needed reconstruction of soft tissue defects for different reasons (Table 3.1). Seven patients underwent a free vascularised tissue transfer in combination with skin transplantation, three patients received only skin transplantation. All donor sites healed without complications.

Table 3.1 Patient characteristics.

Patient	F/M[1]	Age	BMI[2]	Diagnosis
1	M	52	31.4	Burn wounds of the thorax
2	M	43	26.6	Open fracture leg
3	M	32	24.7	Chronic wound ankle
4	F	35	19.7	Defect face after trauma
5	M	75	24.6	Sarcoma of the leg
6	M	36	40.2	Chronic ulcer leg
7	M	70	23.5	SCC leg
8	F	27	27.9	Trauma of the hand
9	M	39	21.2	Leg amputation
10	M	68	25.8	Defect face after SCC[3]
Mean		47.2	26.5	
SEM		5.3	1.9	
Min / Max		27-75	19.7-40.2	

1) F/M; female/male 2) BMI; body mass index 3) SCC: squamous cell carcinoma

Plasma and wound fluid amino acid concentrations

Wound fluid samples were collected on day 2, 5 and 10. At day 10, wound fluid was only harvested from three out of ten patients, and therefore these data were excluded from analysis.

Plasma arginine levels did not differ over time and were similar to healthy humans[31]. Wound fluid arginine levels were lower compared to plasma, however this difference was not significant (Figure 3.1).

Plasma citrulline levels showed no significant difference over time and were similar to healthy humans[31]. In contrast to arginine, wound fluid citrulline levels were significantly higher than plasma levels (Figure 3.2).

Plasma ornithine levels were higher than normal healthy subjects (normal value: 57±1.0 μM[33]). Although an incline was observed from day 2 to 5, no significance was

reached over time. Wound fluid ornithine levels were higher compared with plasma. A significant difference was seen on day 2 (Figure 3.3).

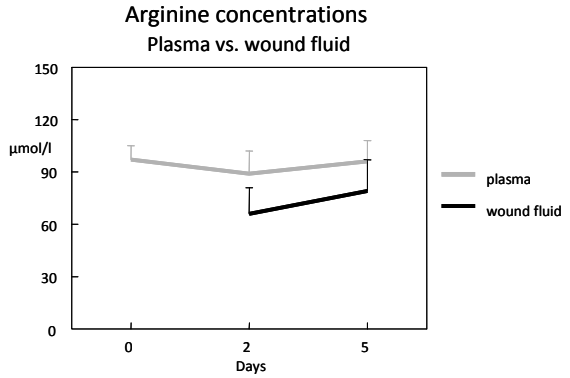

Figure 3.1 Concentrations of arginine in plasma and wound fluid (µM). Plasma samples are obtained pre-operatively, and day 2 to 5 post-operative. Wound fluid samples are obtained day 2 to 5 post-operative. Data are expressed as mean ± SEM, n=10, *p<0.05.

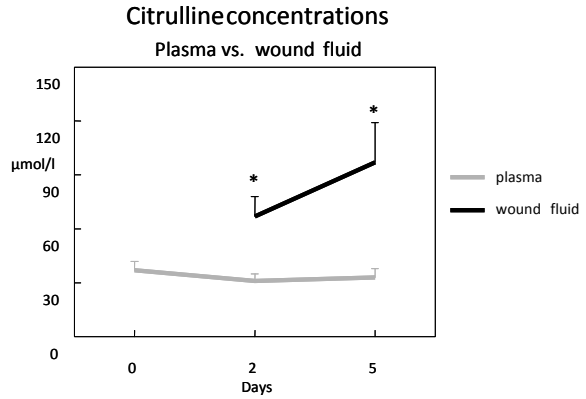

Figure 3.2 Concentrations of citrulline in plasma and wound fluid (µM). Plasma samples are obtained pre-operatively, and day 2 to 5 post-operative. Wound fluid samples are obtained day 2 to 5 post-operative. Data are expressed as mean ± SEM, n=10, *p<0.05.

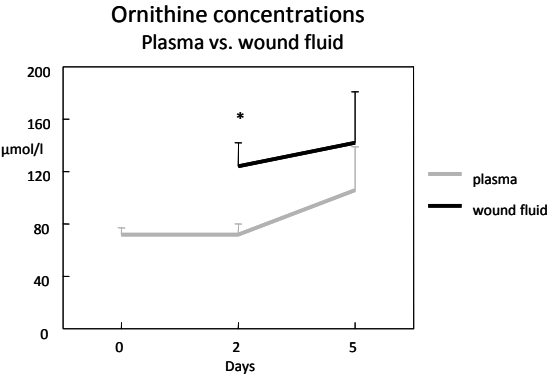

Figure 3.3 Concentrations of ornithine in plasma and wound fluid (µM). Plasma samples are obtained pre-operatively, and day 2 to 5 post-operative. Wound fluid samples are obtained day 2 to 5 post-operative. Data are expressed as mean ± SEM, n=10, *p<0.05.

Plasma and wound fluid NOx

Comparison of plasma and wound fluid nitrite/nitrate levels shows significantly higher NO concentration in wound fluid compared with plasma (Figure 3.4).

Plasma and wound fluid arginase-1

Comparison of plasma and wound fluid Arginase-1 concentrations revealed significantly higher concentrations in wound fluid compared with plasma (Figure 3.5).

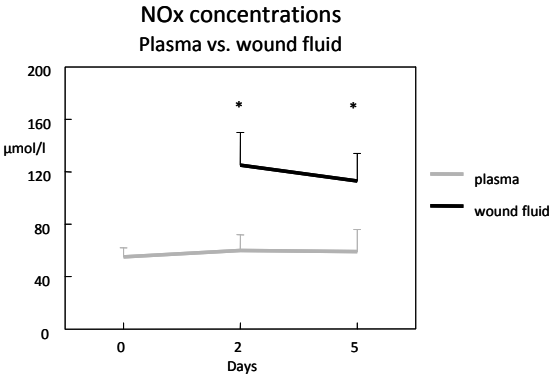

Figure 3.4 Concentrations of NOx (=nitrate+nitrite) in plasma and wound fluid (µM). Plasma samples are obtained pre-operatively, and day 2 to 5 post-operative. Wound fluid samples are obtained day 2 to 5 post-operative. Data are expressed as mean ± SEM, n=10, * p<0.05.

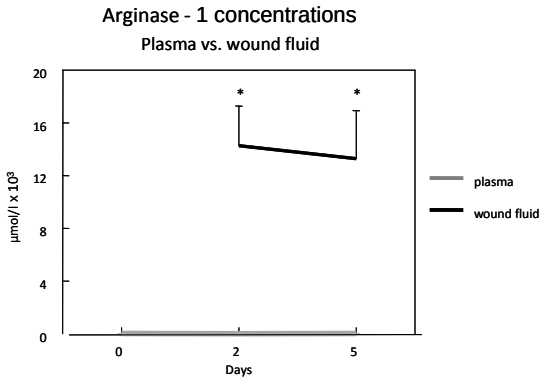

Figure 3.5 Arginase-1 concentrations in plasma and wound fluid (µM). Measurements were performed in plasma on the day of operation (day 0), day 2 and 5 post-operatively. Wound fluid samples were only measured on day 2 and 5 post-operatively. Data are expressed as mean ± SEM, n=10, * $p<0.05$.

Plasma and wound fluid arginase-2

Arginase-2 expression was investigated by Western blot analysis. Using densiometric analysis with LeicaQwin software (Leica Microsystems Switzerland, Ltd.), we could observe increased levels of ARG2 in plasma (Figure 3.6A,B).

Immunohistochemical distribution of iNOS, eNOS and arginase-2 in wounds

The basic histological pattern was similar for all wound sections. Haematoxylin and eosin-stained wound tissue showed a connective tissue matrix filled with fibrin, inflammatory cells, fibroblasts, glandular cells, vessels, smooth muscle cells.

iNOS

In unwounded skin, some constitutive expression was detected in epithelial cells, smooth muscle and endothelial cells. This was in contrast with the abundant staining in wounds (Figure 3.7A, magnification x20), where iNOS was additionally expressed by macrophages, fibroblasts and PMN's (Figure 3.7B, magnification x40). Semi-quantitative analysis showed significantly more positive cells and cell types in wounds compared to normal skin (Table 3.2). Negative control was negative (Figure 3.7C, magnification x20).

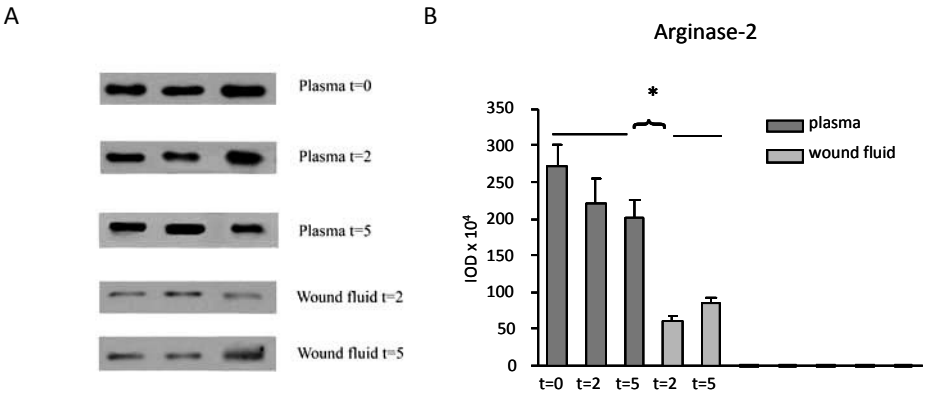

Figure 3.6 Arginase-2 levels in plasma and wound fluid. Enzyme detection by Western Blot (Figure 3.6A). Densiometric quantification of levels, expressed as IOD; mean ± SEM, n=10, * p<0.05 (Figure 3.6B).

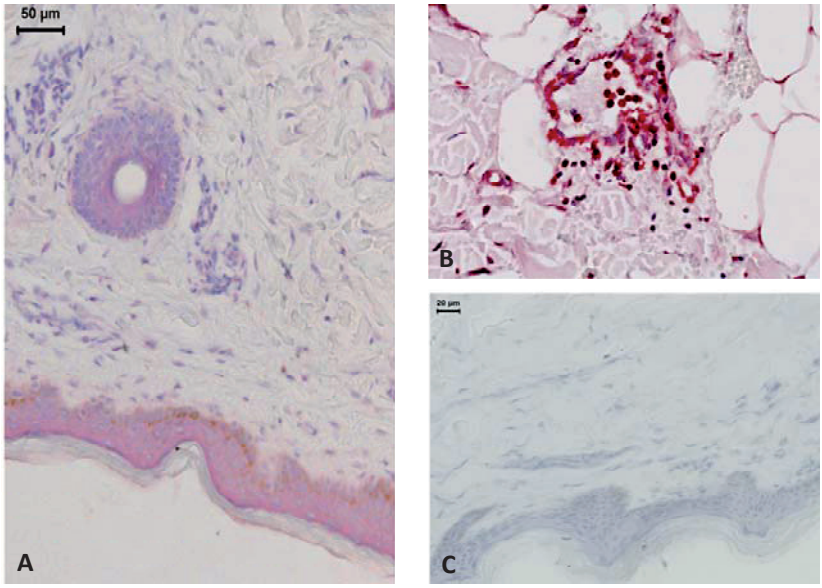

Figure 3.7 iNOS-staining in normal human skin and wounds 10 days post-wounding. Normal skin shows constitutive iNOS-staining in epithelial cells, smooth muscle and endothelial cells (Figure 3.7A; magnification x20). Wounded skin shows similar findings, in addition fibroblasts, PMN's and macrophages stained positive during wound healing (Figure 3.7B magnification x40). Negative control (Figure 3.7C; magnification x20).

eNOS

In unwounded skin, moderate eNOS expression was observed in some of the endothelial cells. In addition, mild eNOS staining was found in keratinocytes and smooth muscle (Figure 3.8A, magnification x20). Semi-quantitative analysis showed equal expression of eNOS in vessels in wounds at all time points $p<0.05$ (Table 3.2). However, in wounded skin the number of blood vessels was significantly increased from day 5 to 10 compared with normal skin (16.1± 1.7 to 28.1± 3.8 vessels/μm^2, $p<0.05$) (Figure 3.8B, magnification x40). Negative control was negative (Figure 3.8C, magnification x20).

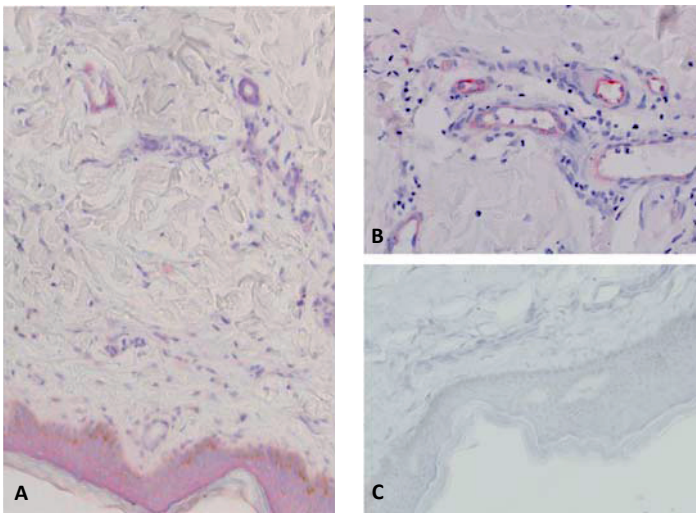

Figure 3.8 eNOS-staining in normal human skin and wounds 10 days post-wounding. Normal skin shows constitutive eNOS-staining in few endothelial cells and keratinocytes (Figure 3.8A; magnification x20). Wound tissue shows positive eNOS-staining predominantly in vessels and some in fibroblasts (Figure 3.8B; magnification x40). Negative control (Figure 3.8C; magnification x20).

Arginase-1

In unwounded skin no arginase-1 staining is observed (Figure 3.9A, magnification x20). In wounded tissue arginase-1 staining is only seen in PMN's (Figure 3.9B: magnification x40). Macrophages are do not show positive staining. Semi-quantitative scoring shows significant more ARG1 expression in PMN's post-wounding compared to normal skin and equal expression of positive PMN's at different days post-wounding (Table 3.2). Negative control was negative (Figure 3.9C, magnification x20).

Table 3.2 Cell types expressing iNOS, eNOS, arginase-1 and arginase-2, normal (unwounded) versus wounded skin.

Cell type	Unwounded	Day 2	Day 5	Day 10
iNOS				
Epithelial cells	0˚	0.75 ± 0.35	0.4± 0.5	0.6± 0.3
Fibroblasts	0˚	0.7 ± 0.3	0.5± 0.3	0.7± 0.4
PMN's	0˚	0.7 ± 0.2	0.35± 0.1	0.25± 0.0
Macrophages	0˚	0.5 ± 0.1	0.7± 0.3	0.7± 0.2
Endothelial cells	0˚	0.8 ± 0.4	0.7± 0.3	0.8± 0.4
Glandular cells	0˚	0.9 ± 0.2	1.0± 0.0	0.65± 0.5
eNOS				
Epithelial cells	1.0 ± 0.0	1.0 ± 0	0.5± 0.5	1.0± 0
Fibroblasts	0	0.3 ± 0.2	0.2± 0.1	0.3± 0.2
PMN's	0	0.2 ± 0.2	0.1± 0.1	0.25± 0.2
Macrophages	0	0	0	0
Endothelial cells	1.0 ± 0.0	1.0 ± 0	1.0 ± 0	1.0 ± 0
Glandular cells	0	0	0	0
Arg-2				
Epithelial cells	0	0	0	0
Fibroblasts	0	0	0	0
PMN's	0˚	1.0 ± 0	1.0 ± 0	1.0 ± 0
Macrophages	0	0	0	0
Endothelial cells	0	0	0	0
Glandular cells	0	0	0	0

PMN's: polymorphonuclear cells; ˚p<0.05 vs. days 2, 5 and 10 post-wounding.
Mean score of enzyme expression on a four point scale; 0: no expression, 1: 1-25% expression, 2: 26-50% expression, 3 : 51-75% expression and 4: 76-100% expression. Mean percentage ± SD is shown.

Arginase-2

In unwounded skin constitutive staining was observed in keratinocytes, smooth muscle and endothelial cells (Figure 3.10A: magnification x20). All cells showed milder expression of arginase-2 in unwounded skin. In wounded tissue (Figure 3.10B: magnification x40) additional staining of macrophages, fibroblasts and PMN's was observed when compared to healthy skin. Semi-quantitative analysis showed significant more cells expressing ARG2 compared to normal skin (Table 3.2). No difference in ARG2-expression was observed at sequential time points during healing. Negative control was negative (Figure 3.10C: magnification x20).

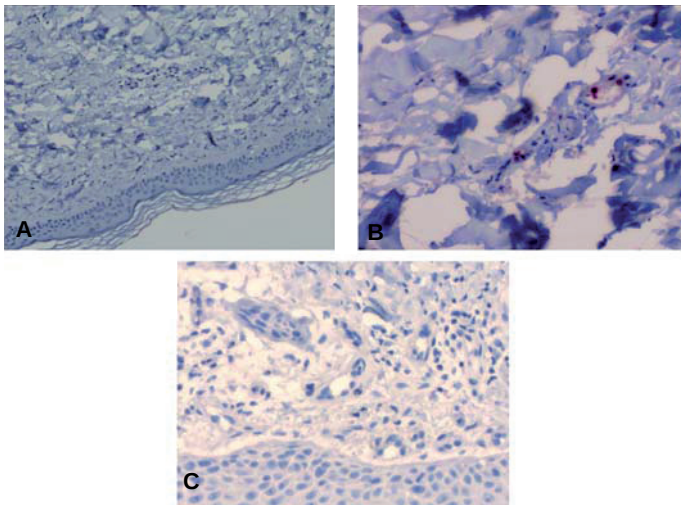

Figure 3.9 Arginase-1 staining in normal skin and wounds 10 days post-wounding. Normal skin shows no arginase-1 staining (Figure 3.9A; magnification x20). In wounded skin arginase-1 staining is only seen in PMN's. Macrophages are not stained (Figure 3.9B; magnification x40). Semi-quantitative scoring shows significant more ARG1 positively stained PMN's in wounds compared to normal skin and equal expression of positive PMN's at different days post-wounding (Table 3.2). Negative control (Figure 3.9C; magnification x20).

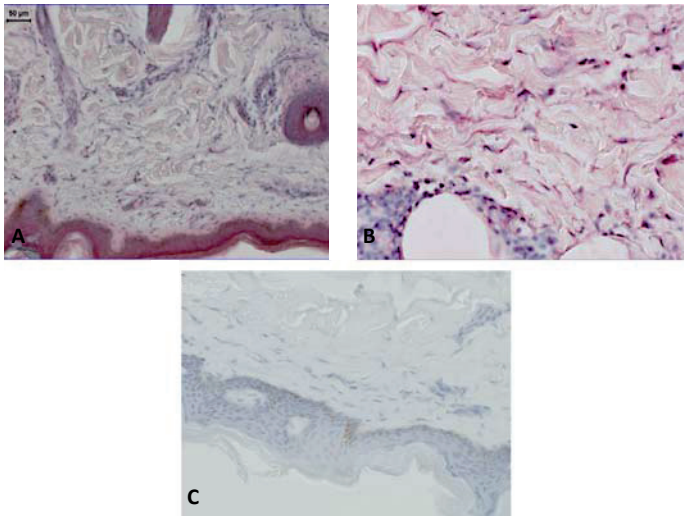

Figure 3.10 Arginase-2-staining in normal human skin and wounds 10 days post- wounding. Constitutive staining was observed in keratinocytes, smooth muscle and endothelial cells of normal skin (Figure 3.10A; magnification x20). In wounds predominantly fibroblasts, macrophages and PMN's stained positive (Figure 3.10B; magnification x40). Negative control was negative (Figure 3.10C: magnification x20).

Discussion

Data from animal studies suggested that arginine metabolism occurs during wound healing. In addition, arginine supplementation in animals and humans appears to improve skin repair. However the mechanisms underlying this effect remain unidentified. To our opinion, this is the first study supporting the role of arginine metabolism in normal wound healing in man. It provides insight in the mechanisms by showing expression of arginine-metabolites and arginine-metabolizing enzymes at different time points during normal healing of surgical wounds, compared with normal skin.

The supporting evidence is shown by increased levels of wound fluid citrulline and NOx when compared with plasma, indicating arginine conversion by NOS[14,34,35]. Moreover, a significant rise of ornithine detected in wound fluid compared with plasma suggests that L-arginine is also metabolised by arginase. Since citrulline and ornithine are not released by protein breakdown, these findings are indicative for arginine metabolism both through NOS and arginase-pathways. This suggestion is also supported by levels of ARG1 and ARG2 detected in wound fluid. Although not significant, arginine levels in wound fluid tend to be lower compared to plasma. This is in line with animal studies[14,35,36], which showed decreased arginine levels in wound fluid when compared with plasma during healing, suggesting arginine consumption at the wound site[34,37-39].

We observed simultaneous increase levels of citrulline and ornithine in wound fluid. Experimental studies show temporal elevation of NOx and ornithine in wound fluid when compared to plasma, suggesting sequential activation of the different arginine metabolic pathways[35,39]. Moreover, others like Finnen et al. suggest a time course requirement for different arginine metabolites, as increased healing upon NO-application in the early healing phase was observed[40,41]. Since the number of sequential samples was limited, we cannot draw conclusions about the time course activation or requirements of arginine metabolism. Unfortunately, more extensive sampling was not ethical for our patients.

The mechanisms by which arginine stimulates cutaneous wound repair is still not completely understood. In the present study, we observed iNOS expression in macrophages, PMN's, fibroblasts, epithelial and endothelial cells upon wounding. In contrast, these cells did not express iNOS in normal skin, indicating wounding activates arginine metabolism. All these cells have specific functions during the complex process of healing and NO has been implicated as a mediator. PMN's and macrophages are needed to debride the wound area and attract other cells important for wound healing. Human neutrophils generate NO[42-44] which modulates their migration[45]. In addition, activated macrophages and neutrophils need arginine for adequate NO-synthesis[46-48]. Moreover, after arginine supplementation, an increased myeloperoxidase-activity in PMN's is observed, that was accompanied by increased NO levels and wound tensile strength[49-51]. Fibroblasts are responsible for collagen formation in granulation tissue, a process essential in tissue repair. In vitro and animal

studies show that NO regulates collagen synthesis in wound fibroblasts[25,38,39,52,53]. Wang et al. showed that fibroblasts derived from human skin express eNOS and iNOS upon stimulation with LPS and INF-γ[54,55]. This is in line with our findings, as eNOS and iNOS were expressed in fibroblasts of wounds at all time points, suggesting NO-mediated collagen formation in wound healing in man (Table 3.2). Neovascularisation is of central importance in wound healing. In vitro studies showed that VEGF, the most potent known angiogenic protein, is induced by NO[56,57]. Inhibition of NO-synthesis impairs VEGF expression and angiogenesis. Moreover, it delays wound healing[58-61]. We observed constitutive expression of eNOS in endothelial cells, which did not alter upon wounding, whereas iNOS was expressed in endothelial cells only after injury. Comparable findings were observed in keratinocytes in present study. They showed a constitutive expression of eNOS and iNOS in normal skin, whereas after injury a more abundant expression of iNOS was noticed. Keratinocytes in normal human skin express all NOS isoforms[61] and iNOS is induced in proliferating keratinocytes upon cutaneous injury in rodents[62]. As all our wounds healed without complications, we speculate that the up-regulation of iNOS/eNOS as seen in the present study implicates that normal human wound healing is NO-mediated, and therefore arginine is required.

Arginase, the other major L-arginine-consuming enzyme, regulates polyamines and proline synthesis through the production of ornithine[1,63]. In addition, arginase regulates NO-formation through substrate competition[64-67]. Previous animal and in vitro studies showed exclusive up regulation of ARG1 during normal wound healing, which was required for collagen formation[25]. Human studies that addressed the role of arginases during wound healing are limited. They described overexpression of arginase in pathological wound states (e.g. diabetes, psoriasis and venous ulcers)[13,16,68]. We detected ARG1 in both plasma and wound fluid. Surgery and sepsis are known to induce elevated plasma levels of ARG1. It has been suggested that ARG1 is released by inflammatory cells, hepatocytes and erythrocytes in order to decrease T-cell immunity[69-72]. In this study, ARG1 levels were significantly higher wound fluid than in plasma, supporting our hypothesis of arginine metabolism through ARG1 in the wound environment. In contrast, we measured higher plasma ARG2 levels compared with wound fluid (Figure 3.6B). ARG2 has a wide tissue distribution, with the highest expression in kidney, prostate and vasculature. In vessels it appears to be involved in the regulation of vascular tone and atherosclerosis[66]. Only Corraliza et al., measured ARG2 in joint fluid from arthritic joints and suggested that this contributed to the disturbed healing process[15]. At present, we do not have an explanation for the elevated levels of ARG2 in plasma and wound fluid observed in this study.

Interestingly we observed coexpression of ARG1 and ARG2 in PMN's, at all time points post-wounding, while no expression was seen in PMN's of normal skin. Coexpression of ARG1 and ARG2 has not yet been reported in PMN's [Munder, 2006, Blood]. However, it has been observed in endothelial cells, where intracellular ARG1 and ARG2 compete for arginine, leading to decreased NO-production. A specific role for arginase isotypes has been suggested; ARG1: ornithine synthesis and ARG2: polyamines

production[73]. Jacobsen suggested that the function of release of arginase from PMN's is to reduce NO-formation by macrophages, endothelial and T-cells[74]. All, these previous findings could apply to PMN function in human wound healing, but the complexity of wound healing in vivo warrants more research. Another interesting finding is that we only observed ARG2 expression in macrophages after injury. Arginase expression by macrophages seems to be species and cell specific as murine macrophages express ARG-1 and -2, rat macrophages ARG1[25,75,76] and human myeloid cells express ARG1[27]. The role of arginase in activated macrophages seems to be down regulation of NO-production through substrate competition which is more pronounced when L-arginine availability is reduced[76,77]. Gotoh et al. suggested early and late arginase expression in macrophages: first ARG2 to produce polyamines for macrophage growth and differentiation and later on ARG1 to produce ornithine for collagen production[77]. It is possible that assessment of ARG in this study was only conducted in the early ARG-expressing phase. Alternatively, different arginase isotypes may play different roles during normal and pathological healing.

Comparable findings were observed in fibroblasts, only expressing ARG2 upon injury. In rats, up-regulation of only ARG1 is detected in fibroblasts after wounding to provide substrate for collagen-synthesis and cell proliferation[25,78].

Furthermore, our study shows ARG2 expression in endothelial cells. Non human endothelial cells express ARG1. Unstimulated human endothelial cells express some ARG2, which increases upon stimulation[79,80]. Arginase seems to be involved in vascular function[65]. In addition, Hui et al. suggested ARG1 is needed for ornithine and ARG2 for polyamines production by bovine endothelial cells[24,73]. Which makes both pathways important for the formation of new blood vessels, an essential process for wound healing.

Finally, expression of ARG2 in epithelial cells of healthy skin was seen, and increased in wounds. The expression of ARG2 in normal skin was already seen by Wohlrab et al. 2002[81]. Present study is the first to show that expression of ARG2 is increased in normal wound healing in man.

In summary, our findings support previous experimental studies suggesting arginine metabolism for an NO-mediated as well as arginase-mediated reparation of injured skin. Our findings suggest a more prominent role of ARG2 in normal healing. As it is known that ARG-1 is more activated in pathological skin healing our finding may have important therapeutic consequences. Inhibition of ARG1 and stimulation of ARG2 during pathological wound healing might be considered in the future. More work remains to be done to translate the needs of injured skin into clinically useful agents.

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Chapter 4

Infected chronic wounds show different local and systemic arginine conversion compared to acute wounds

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Abstract

Background

Several experimental studies have shown the importance of arginine in wound healing. However, little is known about its role in human wound healing. In this study we investigate arginine metabolism in impaired wound healing.

Materials and methods

Twenty patients with chronic wounds and ten patients with acute wounds were included in a prospective study. Amino acids, nitrate/nitrite and arginase concentrations were determined in plasma and wound fluid using High Performance Liquid Chromatography and ELISA. Chronic wounds were divided into two groups: non-infected chronic wounds (n=11) and infected chronic wounds (n=9), based on quantitative bacterial analysis of wound fluid samples.

Results

Plasma arginine levels, next to total plasma amino acid levels, were significantly decreased in patients with infected chronic wounds compared with patients having acute or non-infected wounds. Citrulline and ornithine levels were significantly increased in infected chronic wounds and related to decreased nitrate/nitrite levels, while wound fluid arginine levels were similar in all groups. In addition, wound fluid arginase levels of infected chronic wounds were significantly enhanced.

Conclusions

This study demonstrates that patients with infected chronic wounds have decreased plasma arginine levels, and suggests enhanced arginine conversion in the wound. In contrast to non-infected chronic wounds, arginine seems to be mainly metabolised by arginase in infected chronic wounds. In conclusion, our hypothesis is that impaired wound healing is related to an altered arginine usage.

Introduction

Patients with chronic wounds are a major problem in health care. The resulting prolonged hospital stay generates high costs and impairs the well-being of the patient. The high prevalence of chronic wounds leads to a financial burden in health care, for example in the USA 1.5 to 3 million patients are affected by pressure ulcers[1,2]. Although the development of chronic wounds is multifactorial, nutritional factors have an important role in their development[3]. Supplementation of high amounts of proteins and vitamins to malnourished patients with chronic wounds initially improves wound healing, although complete healing is not observed[4-6].

Recent studies suggest the need of specific amino acids instead of supplementation of high amounts of proteins to stimulate wound healing[7-10]. In this context the amino acids arginine and ornithine are suggested to be of great importance in wound healing[10-14]. The effect of arginine supplementation has been attributed to enhanced synthesis of nitric oxide (NO)[10,12,13,15-18]. NO is a signal molecule involved in immune responsiveness, angiogenesis, epithelialisation and formation of granulation tissue, and has been shown to be critical for healing[19-23].

Ornithine is the precursor for proline, an essential amino acid for collagen synthesis[24] and for polyamines, key components in cell growth and differentiation[14,25-29]. Supplementation of ornithine also has shown to enhance wound healing. The mechanism of action is unclear but it is suggested that that supplementation of ornithine contributes to the synthesis of collagen by increasing the pool of free proline.

Different enzymes are involved in the arginine metabolism in wound healing: NOS and arginase. NOS exists in three isoforms, endothelial NOS, neuronal NOS and inducible NOS, which convert arginine into NO. Inducible NOS (iNOS) is expressed in response to different cytokines and endotoxins and had been shown to be crucial in wound healing[19]. Next to NOS there is arginase, which catalyses the hydrolysis of L-arginine to urea and ornithine. Two different isoforms are identified, differing in cellular distribution: arginase I (AI), mainly localised in liver and arginase II (AII), mainly localized extrahepatic tissue[30]. Next to collagen synthesis, arginase influences immune response[31-33].

From experimental studies it is known that the NOS-pathway dominates the first days of wound healing, whereas the arginase pathway becomes more active after these days[34]. However, little is known of arginine metabolism in acute or chronic wounds in humans. It has been suggested that chronicity in wounds starts with persistent levels of bacteria in the wound tissue, resulting in prolonged elevation of proinflammatory cytokines[35,36]. Although inflammation is part of normal wound healing, an excessive inflammatory response impairs the healing[37].

Based on the above-mentioned findings, we hypothesize that local arginine metabolism is altered in chronic human wounds compared with acute wounds, leading to disturbed wound healing. In addition, we hypothesize that infection may play a role in these changes. Therefore in this study arginine, its metabolites and metabolising

enzymes were measured in plasma and wound fluid of patients with acute and chronic non-infected and infected wounds.

Materials and methods

A prospective study was performed in hospitalised adults having chronic and acute wounds. Patients with chronic wounds were referred to the Chronic Wound Clinic of the Department of Plastic Surgery. Patients underwent screening evaluation, including medical history and physical examination. Nutritional assessment was obtained measuring body weight and length and expressed as Body Mass Index (= weight / lenght^2). Age and sex of the patients and wound parameters were registered. The investigation protocol was approved by the Maastricht Hospital Medical Ethical Board. Each patient signed informed consent.

Acute wounds

Ten patients with an acute surgical wound were included. Details of their characteristics are given in Table 4.1 and 4.2. They were admitted to the hospital one day before surgery and underwent a skin transplantation as part of reconstructive surgery. The donorsite of the skin graft was studied. Exclusion criteria were: age younger than 18 or older than 70 years, kidney or liver failure, pregnancy, active cancer, use of steroids, diabetes mellitus.
Under aseptic conditions and general anaesthesia skin grafts were obtained by using an electric dermatome (Aesculaap®) with a thickness of 0.3 mm. To collect wound fluid, donorsites were covered with a layer of Gordasoft®, then a polyvinylalcohol (PVA) sponge was applied to the wound (Coldex®) and on top of that a transparent dressing (Tegaderm®). The day before wound fluid collection dressing was changed by removing the transparent dressing and the PVA-sponge and reapplying a new sponge and transparent dressing. In this study, wound fluid of acute wounds was collected from the sponge on the second day after surgery. Simultaneously a venous blood sample was drawn from a major vein in the cubital fossa and like the sponge, immediately put on ice.

Chronic wounds

Twenty patients with chronic wounds were included. Chronic wounds were defined as wounds in which the normal healing process is disturbed. Clinical features are: presence of necrotic/unhealthy tissue, lack of adequate blood supply, absence of healthy granulation tissue, lack of reepithelialisation and recurrent breakdown[38]. In this study chronic wounds existing longer than 4 weeks and fulfilling the above mentioned clinical features were studied. Exclusion criteria were haemodynamic instability, sepsis, diabetes mellitus and age younger than 18 or older than 80.

Chronic wounds were treated with Vacuum Assisted Closure® (VAC)-therapy. VAC-therapy consists of a polyurethane foam sponge, which is put in the wound and connected to a pump by a tube. A negative topical pressure is then created in the wound. Wound fluid is drained into a canister attached to the pump. If required, necrotic tissue was removed and VAC therapy was applied. In this study twenty-four hours after initiation of VAC therapy, a single wound fluid sample was collected from the chronic wound. It was drawn from the canister and kept on ice immediately after collection. At the same time a venous blood sample was drawn as described above. Patients with chronic wounds were subdivided into two groups on the basis of results of the bacterial analysis of the wound fluid.

Table 4.1 Main diagnosis and treatment of control patients and wound status of chronic wound patients.

Acute wounds	Main Diagnosis	Operation
1	Cancer of the ear	Reconstruction with free temporal fascia & SSG
2	Tibia fracture	Wound leg: reconstruction ff-gracilis muscle and SSG
3	Sarcoma of the leg	Reconstruction with ff-ALT/SSG
4	Nose contraction after cocaine use	Reconstruction with ff-Radial forearm/SSG
5	Burn wounds back	SSG
6	Venous ulcer leg	SSG
7	Marjolin's ulcer, old burn wounds	Ff-temporal fascia with SSG
8	Crush wound of the hand	Ff-temporal fascia SSG
9	Fracture maleolus	Ff-temporal fascia with SSG
10	Trauma, leg amputation	SSG
Non-infected	Main Diagnosis	Wound status
1	Correction epigastric hernia	W. D. abdomen
2	CABG with Intra Aortic Balloon Pump	W. D. groin
3	Acute Abdominal Aneurysm	W. D. abdomen
4	Cancer rectum	W. D. rectum
5	CABG	W. D. sternum
6	Abdominoplasty	W. D. abdomen
7	CABG	W. D. abdomen
8	CABG	W. D. thorax
9	Abdominoplasty	W. D. abdomen
10	Breast reconstruction with free TRAM	W. D. abdomen
11	CABG	W. D. thorax
Infected		
1	Decubitus sacrum	W. D. sacrum
2	Decubitus sacrum	W. D. sacrum
3	Rectal Cancer	W. D. rectum
4	CABG	W. D. thorax
5	Thoracic aneurysm	W. D. abdomen
6	Breast reconstruction with TRAM	W. D. breast
7	Calcaneus fracture, removal OSM	W. D. maleolus
8	Wound infection after fall	W. D. lower leg
9	CABG	W. D. thorax

SSG, split skin graft; Ff, Free flap; CABG, Coronary artery bypass surgery; W.D, wound dehiscence; TRAM, transverse rectus abdominis musculocutaneous flap; OSM, osteosynthesis material.

Table 4.2 Patient characteristics.

Group	F:M	Age		BMI		Plasma ESR		Plasma CRP		Plasma Albumin	
		yr		Kg/m^2		(Ref 0-12mm)		(Ref 2.0-9.0 mg/l)		(Ref 35-55 g/l)	
		Mean	SEM	Mean	Min / Max	Mean	SEM	Mean	SEM	Mean	SEM
Acute wounds	2:8	47.2	5.3	26.5	20-40	12.0	3.6	70.2	17.8	37.9	5.5
Chronic wounds											
Non-infected	4:7	58.3	2.7	26.4	21-41	67.8*	7.5	85.6	21.2	20.8*	1.5
Infected	6:3	61.0*	3.8	26.3	17-38	65.6*	15.0	92.2	15.5	21.5*	1.6

BMI, Body Mass Index; ESR, Erythrocyte Sedimentation Rate; CRP, C-reactive protein. All values are expressed as means ± SEM, except for BMI which is expressed as mean, min and max values.
* $p<0.05$ compared to the acute wound group.

Bacterial analysis

Quantitative and specific bacterial analysis were obtained from the wound fluid of acute and chronic wounds. After collection a wound fluid sample was immediately sent to the laboratory of microbiology of our hospital for quantitative and specific bacterial analysis. A negative culture was defined as less than 10^3/ml Colony Forming Units (CFU) and a positive culture as more than 10^3 CFU/ml[39].

Sample processing and analysis

Blood was centrifuged at 4°C during 10 minutes at 4000 rpm within one hour after sampling. After centrifugation, 500 µl of plasma was deproteinized using 20 mg dry sulphosalicylic acid (SSA), vortexed and frozen in liquid nitrogen. Samples were stored at -80 degrees Celsius until analysis. Wound fluid from chronic wounds was collected from the canister of the VAC-system. To obtain wound fluid from acute wounds, sponges were centrifuged at 4°C during 10 min at 11.000 rpm. After centrifugation, 500 µl of wound fluid was treated similar to the plasma.

Biochemical analysis

Amino acid concentrations

Plasma and wound fluid amino acids were determined using a fully automated HPLC system as described previously[40]. Nitrate / nitrite concentrations in plasma and wound fluid were also determined using HPLC[41]. The total sum of all amino acids was calculated by adding the concentrations of all individual amino acids (SumAA).

Erythrocyte Sedimentation Rate, C-Reactive Protein and albumin

ESR was measured using the Westergren method (mm/ 1st hour). Albumin was measured with standard laboratory tests and expressed as g/l. CRP analysis on the Synchron LX®20 System, a routine clinical chemistry analyser from Beckman Coulter, is

based on immunoturbidimetry, using a polyclonal anti-CRP antibody (Beckman Coulter, Inc. Fullerton, CA, US). The detection limit of the assay is 1.0 mg/l.

Elisa for arginase I

Plasma and wound fluid arginase levels were measured using sandwich ELISA[42]. Standard, primary and secondary antibodies were kindly provided by HBT (Uden, the Netherlands). In short, a 96-wells plates were coated overnight at 4°C with 100 µl of the purified anti-human liver-type arginase monoclonal IgG (Mo6G3). Plasma samples were prediluted 1:2 in dilution buffer (1x PBS-0.1% BSA) and wound fluid samples 1: 400. Bound arginase was detected with a second biotinylated antibody (Mo9C5), followed by peroxidase-conjugated streptavidin and TMB.

Statistical analysis

A power analysis was performed for this study: Based on previous (pilot) studies we considered a difference of 40 mmol/l between plasma and wound fluid amino acid levels as relevant. A total number of 20 patients was needed to show a significant difference. Data are expressed as mean ± SEM. Amino acid concentrations were expressed as µM. To compare the plasma values from chronic wounds with plasma values from acute wounds the non-parametric Mann-Whitney test for independent groups was performed. The same was done for wound fluid. To compare plasma with wound fluid within one group a the non-parametric Wilcoxon test for dependent groups was used. In all cases $p<0.05$ was selected as criterion for statistical significance. The statistical package for social sciences (SPSS) program was used for statistical analysis.

Results

Patient and wound status

In Table 4.2 patients nutritional state and disease activity, assessed by BMI, Albumin, ESR, and CRP are shown. There were no differences in BMI and CRP levels between the groups. In contrast ESR and albumin levels differ. ESR was significantly lower in patients with acute wounds and albumin was significantly higher in this group compared with chronic wounds, indicating less disease activity in patients with acute wounds. A small difference in age between the patients with acute wounds and those with chronic infected wounds was observed.

Acute wounds showed no signs of infection and in addition no bacteria were cultured from the wound fluid of these wounds. Eleven chronic wounds showed a negative bacterial culture, further referred to as the *non-infected chronic wound*s. Nine chronic wounds showed a positive bacterial culture*: the infected chronic wound*s. More than

one bacterial strain was found; following species were primarily cultured: Pseudomonas aeruginosa, Staphylococcus aureus, E-coli, Proteus mirabillis.

Amino acid concentrations in plasma and wound fluid

To identify the specific influence of the presence of different wound types on circulating amino acid levels we measured plasma amino acids levels in all groups. The results revealed significantly decreased plasma arginine levels in patients with infected chronic wounds compared with patients with acute and non-infected wounds (Figure 4.1A). Plasma citrulline and ornithine levels did not differ between the groups. Table 4.3 and 4.4 show the analysed amino acids in plasma and wound fluid. The plasma SumAA (Figure 4.2A), histidine, alanine, valine and methionine concentrations (Table 4.3) were significantly lower in patients with infected chronic wounds compared with patients suffering from acute or non-infected wounds.

Table 4.3 Plasma amino acid concentrations in control patients and patients with chronic wounds.

Plasma	Acute wounds		Non-infected wounds		Infected wounds	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
GLU	85	8	84	12	78	8
ASN	52	5	47	4	45	3
SER	122	15	101	6	94	6
GLN	683	60	647	30	563	59
HIS	75	8	73	4	57*	5
GLY	250	35	234	19	217	28
THR	131	15	128	15	106	9
ALA	500	79	349*	25	272*	30
TAU	70	7	69	10	59	13
aAB	19	4	15	2	18	4
TYR	59	4	58	6	54	5
VAL	240	20	225	24	173*	15
MET	40	5	24*	3	21*	2
ILE	87	8	85	9	69	9
PHE	67	7	64	4	58	4
TRP	42	4	42	4	32	3
LEU	144	14	136	16	109	15
LYS	199	21	164	21	166	11
SUM AA	3056	250	2738	115	2350*	107

All values are means ± SEM; AA, amino acids. * $p<0.05$ significantly different from the controls subjects

To investigate local amino acid metabolism in wounds, we measured wound fluid amino acid levels of each group. Figure 4.1B shows similar wound fluid arginine levels in acute and chronic wounds. Citrulline and ornithine levels in wound fluid of infected wounds were significantly increased compared with acute and non-infected wounds. In addition, SumAA (Figure 4.2B), glutamate, histidine, glycine, alanine, taurine, tyrosine, valine, methionine, isoleucine, phenylalanine, leucine and lysine (Table 4.4)

were significantly higher in infected chronic wounds compared with acute and non-infected wounds.

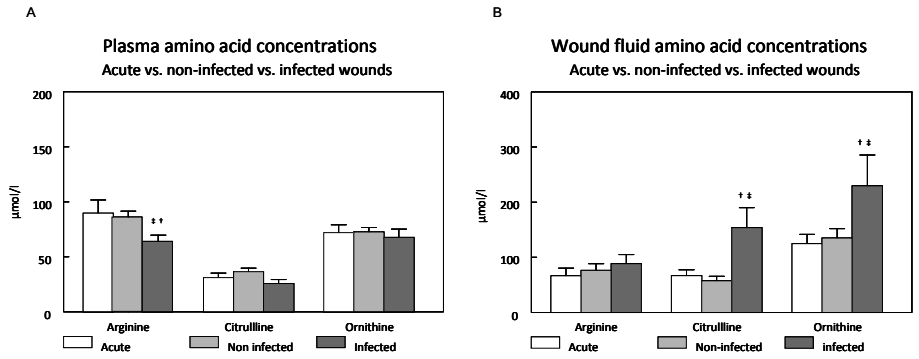

Figure 4.1 Plasma and wound fluid amino acid concentrations of the acute, non-infected and infected chronic wounds are shown. Data represent mean ± SEM. † p<0.05 versus non-infected wounds; ‡ p<0.05 versus acute wounds.

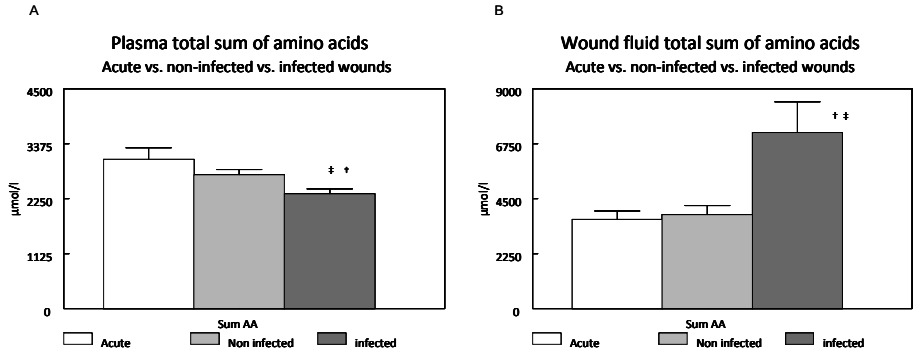

Figure 4.2 Plasma and wound fluid total sum of amino acids (SUM AA) are shown. † p<0.05 versus non-infected wounds; ‡ p<0.05 versus acute wounds.

Table 4.4 Wound fluid amino acid concentrations in control patients and patients with chronic wounds.

Wound fluid	Acute wounds		Non-infected wounds		Infected wounds	
	Mean	SEM	Mean	SEM	Mean	SEM
GLU	179	25	206	42	460*º	116
ASN	51	6	53	9	45	23
SER	265	39	172	26	317	93
GLN	535	61	551	75	589	94
HIS	94	13	100	14	167*º	36
GLY	344	52	393	44	641*º	136
THR	170	19	171	23	291	80
ALA	503	44	476	53	786*º	155
TAU	281	45	212	40	702*º	229
AAB	15	3	30	14	32	9
TYR	67	8	91	12	311*º	104
VAL	233	24	302	38	502*º	93
MET	38	6	34	4	77*º	18
ILE	91	9	126	17	281*º	59
PHE	91	11	119	16	280*º	58
TRP	38	4	41	6	64	16
LEU	178	19	223	40	561*º	122
LYS	222	26	276	37	631*º	121
SUM AA	3653	357	3848	383	7208*º	1331

All values are means ± SEM; AA, amino acids. * $p<0.05$ significantly different from the controls subjects; º $p<0.05$ significantly different from the patients with non-infected chronic wounds.

To address wound specific influences on local amino acid metabolism we compared wound fluid amino acid levels with plasma amino acid levels within the groups. Ratio's were calculated by dividing wound fluid by plasma amino acid levels of which a ratio <1 indicates lower wound concentrations relative to plasma levels (Figure 4.3). Comparison of arginine ratios revealed no significant differences between the groups. However, lower arginine ratios were observed in wound fluid of acute and non-infected chronic wounds (ratio <1), compared to infected wounds (ratio >1). Wound fluid citrulline and ornithine ratios, were higher than plasma for all groups. With the most significant increase of wound fluid citrulline in infected chronic wounds.

Nitrate / nitrite concentrations in plasma and wound fluid

Analysis of the nitrite and nitrate concentrations (NOx), used as an index of NO synthesis ,demonstrated significantly decreased NOx levels in wound fluid of infected chronic wounds compared with acute wounds (Figure 4.4B) . Comparison of plasma NOx revealed no differences between all groups (Figure 4.4A).

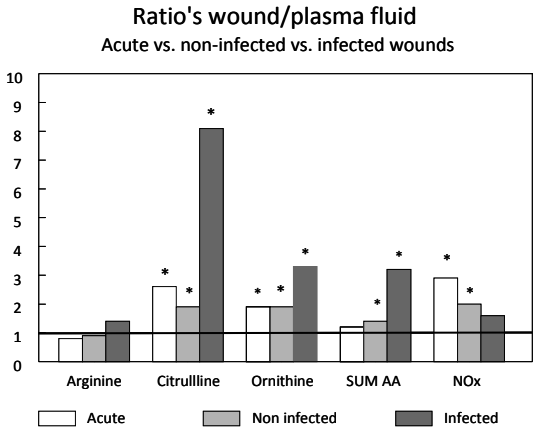

Figure 4.3 Ratio's of plasma and wound fluid arginine, citrulline, ornithine, total SUM of amino acids and sum of nitrite and nitrate (NOx). Wound fluid values are divided by plasma values. * wound fluid/plasma within one group : p<0.05.

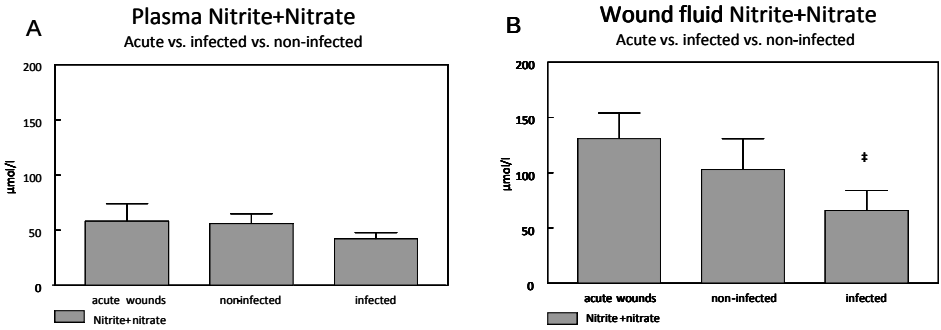

Figure 4.4 Sum of nitrite and nitrate concentrations (NOx) in plasma and wound fluid of the acute, non-infected and infected chronic wounds are shown. Data represent mean ± SEM. ‡ p<0.05 versus acute wounds.

Arginase-1 levels in plasma and wound fluid

No differences were observed in plasma arginase levels between the groups (Figure 4.5A). Comparison of wound fluid with plasma levels showed a substantial increase of wound fluid arginase levels of all groups, indicating arginase release locally in the wounds (Figure 4.5B). This finding was most striking in infected chronic wounds.

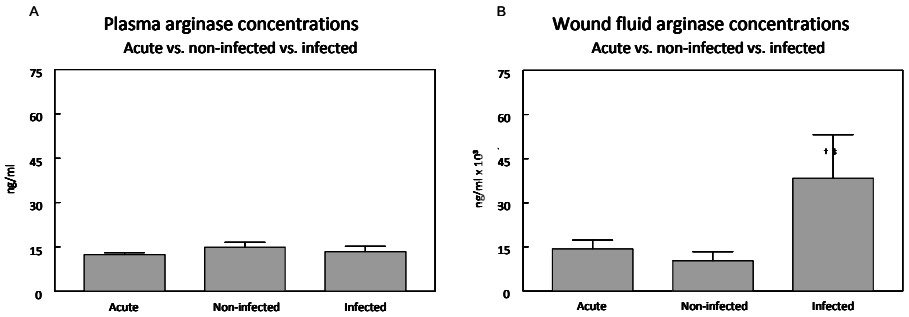

Figure 4.5 Plasma and wound fluid arginase concentrations of the acute, non-infected and infected wounds are shown. Data represent mean ± SEM.

Discussion

This study shows that infected chronic wounds have increased citrulline, ornithine and arginase-1 levels and decreased nitrite/nitrate levels compared to acute and non-infected chronic wounds, indicating an altered arginine conversion. In addition, these patients had lowered plasma arginine levels, suggesting enhanced use of arginine.

Previous studies with rodents suggest the local use of arginine by NOS and arginase in acute wound healing[34,43]. Patients in our study, with acute wounds demonstrated decreased wound fluid arginine levels compared with plasma (ratio <1), indicating the use of arginine. As citrulline and NOx levels were clearly increased in wound fluid compared to plasma, this was indicative for NO synthesis by NOS in acute wounds[43,44]. Furthermore, our data show increased wound fluid ornithine and arginase-1 levels compared with plasma in acute wounds. Ornithine is the precursor for proline, which is needed for collagen and polyamine synthesis. These findings suggest arginine metabolism via arginase[45,46]. The acute wounds in this study (donorsites of SSG) all healed within two weeks (data not shown). Therefore these data suggest activation of both pathways of the L-arginine metabolism during normal acute wound healing in humans possibly similar to experimental studies.

Next, arginine conversion was studied in chronic wounds. As infection complicates the course of wound healing and successful closure of wounds is related to a low bacterial count[37], we studied whether this was related to an altered arginine metabolism. Therefore we subdivided the chronic wounds into two categories: non-infected and infected.

Non-infected chronic wounds show increased levels of wound fluid ornithine and citrulline compared with plasma (Figure 4.3). These increased levels are to be explained by conversion of arginine into its metabolites. Proteolysis, which may be activated in chronic wounds, can not be an explanation for these increased levels

because ornithine and citrulline are not incorporated in proteins. Therefore these findings suggests activation of both NOS and arginase-pathways in non-infected chronic wounds, as observed in the acute wounds. However, no significant difference between plasma and wound fluid arginine (ratio=1) is observed, suggesting arginine is not used. The use of vacuum therapy in these chronic wounds, contrary to acute wounds, could camouflage arginine metabolism. It is known that vacuum improves wound healing, however the exact mechanism remains unknown[47,48]**.** The continuous negative pressure created by vacuum causes a flow of extravasated plasma to the wound environment and therefore leads to a constant supply of amino acids from plasma. An equilibrium between plasma and wound fluid amino acid levels is expected if no metabolism would take place. In case of utilization, vacuum may cause equilibration of levels and therefore may explain the comparable arginine plasma and wound levels. However, in case of production vacuum is not able to camouflage increased levels of amino acids in wound fluid. As citrulline and ornithine are increased we therefore think that arginine is utilized in non-infected chronic wounds and that the use of vacuum camouflages decreased arginine levels.

Infected chronic wounds showed some clear differences in amino acid concentrations. First, decreased plasma arginine and SumAA levels are observed ($p<0.05$) in patients with infected chronic wounds compared with patients with non-infected or acute wounds. Decreased plasma arginine levels have been previously observed in patients with burn wounds, trauma and patients undergoing surgery[49-51] and were attributed to an increased metabolic demand due to increased systemic inflammatory response. However, our present results show that patients with infected chronic wounds have decreased plasma AA, while no differences in systemic inflammatory response, expressed by ESR, CRP and albumin, are observed compared with patients having non-infected chronic wounds. Also, for other potentially influencing factors such as vascular disease or cancer treatment e.g. radio- or chemotherapy, infected and non-infected groups are comparable. In addition, both groups show BMI's >20, indicating normal weight[52,53]. In combination with normal dietary intake, makes chronic malnutrition unlikely (Table 4.2). Therefore these findings suggest that the observed systemic difference in plasma amino acids is due to the infection of the wound, which is also supported by the findings that no plasma amino acid differences are observed between non-infected chronic and acute wounds, both showing no signs of infection in the wounds.

Infected chronic wounds showed a wound/plasma arginine ratio >1, suggesting accumulation of arginine locally in the wound instead of use (Figure 4.3). This observation seems to conflict with our hypothesis of utilization of arginine. However, as in non-infected wounds vacuum may camouflage levels of substrates being used as there is a continuous drainage and thus supply of arginine from the plasma pool. Secondly, the higher arginine ratio in infected wounds compared to non-infected wounds may be explained by the higher amount of protein breakdown in the wounds which will result in a general higher level of amino acids included in protein[54-56]. The

finding of less arginine in wound fluid (arginine ratio = 1.4) compared with other amino acids incorporated in protein, e.g. serine (ratio 3.8), lysine (ratio 3.8) and the SumAA ratio (3.2), supports the hypothesis of use of arginine in these wounds.

Another argument for arginine utilization is the significantly higher wound fluid ornithine and arginase-I levels in infected wounds compared to acute and non-infected wounds (Figure 4.1B + 4.5B). As mentioned earlier, ornithine cannot be released by proteolysis and therefore we think this finding reflects metabolism. Although the synthesis of ornithine is essential and supplementation beneficial to wound healing[46,57,58], enhanced expression of arginase has been previously related to impaired healing in diabetic and venous ulcers[59,60] . In addition, it has been shown that too much arginase decreases arginine availability for NO production by substrate competition[29,61].

Finally, wound fluid NOx levels of infected chronic wounds were decreased compared to acute and non-infected wounds, while no difference in plasma NOx were observed between the groups (Figure 4.4A). This indicates decreased NO synthesis in the infected chronic wound. NO is produced by macrophages and elicited polymorphonuclear leucocytes to destruct pathogens. Overproduction of NO may also exert cytotoxic effects on structural elements in wound healing[62]. However, deficient NO production may be responsible for a defective defence barrier. In addition, reduced NO levels could be disadvantageous for wound healing, as NO is needed for numerous processes essential to wound healing[63,64] like angiogenesis and collagen formation.

In line with decreased NOx levels we would expect citrulline to be decreased too. An explanation for the unexpected high citrulline concentration might be the direct conversion of arginine into citrulline by the enzyme arginine deiminase (ADI) expressed by Pseudomonas aeruginosa and E-coli bacteria. These bacteria were cultured in wound fluid of infected wounds and can use arginine to generate ATP[65-67]. Summarizing, we postulate that in infected chronic wounds arginine is increasingly converted into ornithine and that there is decreased NO production. Whether bacterial colonisation is the cause or the effect of the altered arginine metabolism remains speculative.

Although the exact role of the other changes in amino acid levels observed is unknown and literature is scarce some amino acids may be important for wound healing. It is beyond the scope of this article to extensively discuss all these altered amino acid levels. In summary, the most prominent observation is the general increase in amino acids in the infected wounds (Table 4.4) explained as stated earlier by proteolysis. The increased pool of free amino acids may be needed for protein synthesis and other metabolic processes. Glutamate e.g. as a precursor for ornithine and proline synthesis. BCAA's (valine, leucine and isoleucine) as precursors for protein synthesis and as oxidative fuels[68-75].

Our main aim was to investigate the differences in arginine metabolism in non-infected and infected chronic wounds, as the importance of arginine in wound healing is extensively studied in animal studies. In our study, clear differences are observed in

infected chronic wounds. This is an interesting finding as these wounds only differ in infection status compared with non-infected and acute wounds. We postulate that enhanced expression of arginase-1 and the presence of arginine-metabolising bacteria during infection, decrease NO synthesis which could be related to impaired healing of infected chronic wounds. At the moment studies are conducted to investigate arginine metabolism in acute and chronic wounds over time.

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Chapter 5

Oral arginine supplementation and the effect on skin graft donor sites; a randomized clinical pilot study

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Abstract

Background & Aims
Although arginine has been shown to improve healing in rodents and in small induced wounds in healthy volunteers, little is known about the effects of arginine supplementation on healing of clinically relevant surgical wounds.

Methods
We studied 18 patients in a double blind randomized pilot study (12 males, 6 females), who underwent skin transplantation as part of reconstructive surgery. Patients were randomly assigned to receive arginine (n=8) or placebo (n=10) supplementation as an enteral dose of 36.2 grams of L-arginine-HCl or an isocaloric amount of placebo (51.2 grams alanine), respectively. Wound healing was evaluated at the donor sites of skin grafts by measuring angiogenesis, re-epithelialization and neutrophil count. Arginine metabolism was studied by measuring plasma and wound fluid amino acid concentrations.

Results
Our results show that none of these parameters were significantly different between the oral arginine supplementation group and the placebo group.

Conclusion
In conclusion, enteral arginine supplementation does not improve wound healing of skin donor sites.

Introduction

Although nutrition is an extrinsic factor regulating wound healing, nutritional supplementation for the modulation wound healing remains experimental[1,2]. Until now no studies investigated the effect of supplementation of pharmaconutrients to relatively healthy patients with clinically relevant surgical wounds. This is of great importance in e.g. burn wound treatment; speeding up donor site healing can result in shorter hospital stay leading to increased well-being of the patient and lower costs of healthcare.

Arginine has been shown to have an important role in experimental wound healing. Excluding arginine from the diet leads to decreased wound healing[3] while supplementation of arginine increases wound collagen accumulation, wound breaking strength and reepithelialization in rodents[4-8]. Beneficial effects of arginine supplementation are attributed to enhanced local nitric oxide (NO) production, a signal molecule needed to mediate processes essential for wound healing (e.g. vascular relaxation, platelet aggregation, neurotransmission and non-specific host defense)[9-13]. Although the exact role of NO remains unclear[14,15], different studies show arginine is a potent immunomodulator; it improves on patient's immune state: improvement of mitogen-stimulated lymphocyte proliferation in burned children[16], nitrogen retention, decreased hospital stay and wound infection rates[17-19]. Furthermore, NO is a key mediator in angiogenesis; iNOS-derived (induced nitric oxide synthetase) NO induces VEGF (vascular endothelial growth factor), the most potent known angiogenic protein[20-22]. In addition, Noiri et al. observed that inhibition of NO synthesis blunted VEGF-stimulated cell migration. Comparable results were published by Most et al., showing that i-or eNOS knockout mice exhibited impaired VEGF expression and delayed wound healing[23] .

Arginine has been evaluated using nutritional formulas containing other nutrients like glutamine, nucleotides and ω-3 fatty acids. From these studies, it was not possible to attribute the positive effects of these nutritional formulas strictly to arginine[24-26]. Only Van Bokhorst et al. studied the isolated role of peri-operative arginine supplementation as pharmaconutrient[18]. Moreover, this study only evaluated postoperative morbidity as parameter of wound healing. Although, oral arginine supplementation was studied in healthy volunteers with experimental wounds, showing increased wound collagen synthesis, so far, no clinical studies have investigated the effect of arginine supplementation on clinically relevant wound healing. These prior results prompted us to conduct a pilot study that investigates the effects of arginine supplementation on the healing of skin graft donor sites in well-nourished patients. In addition we tried to clarify the way arginine supplementation affects conversion of arginine in wounds.

Materials and methods

Subjects

Eighteen patients (12 males, 6 females) undergoing skin transplantation as part of reconstructive surgery participated in this double-blind randomized study. Age ranged from 25 to 70 years (mean±SEM: 46±17). Mean body mass index and weight loss within six months prior to surgery was measured to determine clinical depletion according to the Espen guidelines[27]. Exclusion criteria were age younger than 18 or older than 70 years, kidney or liver failure, pregnancy, use of steroids, immune deficiency diseases, and diabetes mellitus. The study protocol was approved by the Ethics Committee of Maastricht University Hospital, and informed consent was obtained for each subject.

Patients were randomly assigned to arginine (n=8) or placebo treatment (n=10), by an independent dietician, using numbered envelopes. Based on literature at the initiation of the study, the highest tolerable amount of arginine was administered[4,5]. Supplementation was given during five days peri-operatively to evaluate the different processes during the initial phases of wound healing (inflammatory and proliferative phase). Patients received either 36.2 grams of L-arginine-HCl (26 grams of free arginine) or an isocaloric amount of placebo (51.2 grams alanine). We chose isocaloric, because we were not able to make solutions that were isocaloric and isonitrogenous without compromising the study design. To make the solutions isocaloric and isonitrogenous, they would not be isovolumetric and this would make a double blind study impossible. L-arginine-HCl or alanine was dissolved in 600 ml of commercially available sugar-free lemonade (Slimpie®) to ameliorate the taste. Patients drank three doses of 200 ml per day for 5 days, in the presence of the investigator. Arginine or placebo (Bufa, Uitgeest, The Netherlands) supplementation was started the day before the skin graft was harvested and continued until the fourth day after surgery. No arginine/placebo drink was administered on the day of operation. Oral food intake was allowed as desired. Samples were collected prior to supplement ingestion.

Wound model

All clinical wound procedures were performed in the Department of Plastic Surgery, University Hospital Maastricht, The Netherlands. Under general anesthesia and aseptic conditions, split skin grafts were obtained using an electric dermatome (Aesculaap®) with a thickness of 0.3 mm. The donor sites were used to evaluate wound healing. Wound fluid was collected from these donor sites by covering it with a layer of Gordasoft®, followed by a polyvinyl alcohol sponge (Coldex®) and a transparent dressing (Tegaderm®) on top.

Twenty-four hours before sampling the dressing was changed by removing the transparent dressing and the sponge, followed by reapplying a new sponge and transparent dressing. From each patient wound samples were taken on day 2, 5 and

10. After the sponge was removed, it was immediately put on ice. From the central part of the donor site 3-mm punch biopsies were taken. Before the excision of the biopsies, lidocaine was locally injected. Subsequently a venous blood sample was drawn from a major vein in the cubital fossa. The surfaces of all donor sites were measured.

Sample processing and analysis

Heparinized blood was centrifuged at 4°C for 10 minutes at 4000 rpm within one hour after sampling. After centrifugation, 500 µl of plasma was deproteinized using 20 mg dry sulphosalicylic acid (SSA), vortexed and frozen in liquid nitrogen. Samples were stored at -80 degrees Celsius until analysis. Wound fluid from acute wounds was obtained by centrifuging the sponges for 10 min at 4°C (11.000 rpm). After centrifugation, 500 µl of wound fluid was treated similar to the plasma. The recovery of fluid from the sponges was validated and found to be constant.

Evaluation of wound healing

In order to study the wound healing, donor site biopsies were obtained at identical time points: day 2, 5 and 10. These time points were chosen to reflect the inflammatory phase at day 2, the proliferative phase at day 5 and reepithelialization on day 10. Biopsies were fixed in 4% formaldehyde, processed by routine histological procedures and embedded in paraffin. Four µm sections were subsequently obtained from each paraffin block.

Immunohistochemical assessment of neutrophils and angiogenesis

Sections were initially stained with haematoxylin and eosin. Polymorphonuclear neutrophils (PMN's) were counted in wound biopsies of day 2, 5 and 10 post-surgery in sections as a marker of inflammation.
We objectified angiogenesis by micro vessel density (MVD), as described previously by Hillen et al. and Baeten et al.[28,29] in wound of day 2, 5 and 10 post-surgery. Immunolabeling of mouse-anti-human alpha smooth muscle actin (ASMA; Dako Laboratories, Carpinteria, CA, USA) and mouse-anti-human CD31 (Dako Laboratories Carpinteria, CA, USA) was used to stain vessels on separate days. The assessments were performed by two separate blinded investigators.

Reepithelialization

Standardized photographs of the donor sites with measuring scales were taken on days 5 and 10 (Canon EOS 300 with macro lens, 1:1). Two blinded independent observers measured the surface of reepithelialized skin by planimetry using the morphometric program ImageJ[30] (W. Bailer, Hagenberg, Austria). The area with reepithelialized skin

was expressed as a percentage of the total wound area (central part). These type of wounds usually heal within 10 days, i.e. reepithelialization percentages of approximately 80%[31,32]. A quantitative bacterial analysis was performed of wound fluid samples, expressed as colony forming units (CFU).

Biochemical analysis

Amino acids measurement in plasma and wound fluid

Arginine, citrulline and ornithine concentrations were measured in plasma and wound fluids of all patients. Plasma and wound fluid amino acids were determined using a fully automated HPLC system as described previously[33].

Nitrite/nitrate determination in plasma and wound fluid

To study production of NO by Nitric Oxide Synthetase (NOS) we measured the concentration of the indicators of NO production (nitrite and nitrate) and expressed these results as NOx (=sum of nitrite and nitrate). NOx concentrations in plasma and wound fluid were also determined using HPLC[33,34].

Arginase-1 concentrations in plasma and wound fluid

Arginase-1 concentrations were determined in plasma and wound fluid samples of all patients to objectify the involvement of this arginine-metabolizing enzyme. A sandwich ELISA method was used to measure arginase levels[35]. Standard, primary and secondary antibodies were kindly provided by HBT (Uden, The Netherlands). In short, a 96-wells plate was coated overnight at 4°C with 100 µl of the purified anti-human liver-type arginase monoclonal IgG antibody (Mo6G3). Plasma samples were prediluted 1:2 in dilution buffer (1x PBS-0.1% BSA) and wound fluid samples 1: 400. Samples were added to the wells. Bound arginase was detected with a second biotinylated antibody (Mo9C5), followed by peroxidase-conjugated streptavidin and TMB.

Statistical analysis

Data were expressed as means ± SEM. Amino acid concentrations were expressed in µmol/l. Repeat-measure ANOVA was used to compare both groups. A 95% confidence interval was used to determine significance. The statistical package for social sciences (SPSS) program was used for statistical analysis.

Results

Patient characteristics

Patient characteristics are shown in Table 5.1 and 5.2.

Table 5.1 Patient characteristics (gender, age, BMI).

Group	F:M	Age yr		BMI Kg/m²	
		Mean	SEM	Mean	Min/ max
Controls	8:2	47	6	26.5	20 / 40
Suppletion	4:4	49	6	27.6	20 / 35

BMI, Body Mass Index. All values are expressed as means ± SEM, except for BMI which is expressed as mean, min and max values.

Table 5.2 Patient characteristics (diagnosis, operation).

Controls	Main Diagnosis	Operation
1	Cancer of the ear	Reconstruction with free temporal fascia & SSG
2	Tibia fracture	Open wound leg: reconstruction with ff-Gracilis muscle and SSG
3	Sarcoma of the leg	Reconstruction with ff-ALT and SSG
4	Nose contraction after cocaine use	Reconstruction with ff-Radial forearm / SSG
5	Burn wounds back	SSG
6	Venous ulcer leg	SSG
7	Marjolin's ulcer, old burn wounds	Ff-temporal fascia with SSG
8	Crush wound of the hand	Ff-temporal fascia SSG
9	Fracture maleolus	Ff-temporal fascia with SSG
10	Trauma, leg amputation	SSG
Suppletion	Main Diagnosis	
1	Scar formation after burn wounds	SSG
2	Open wound after fracture of tibia	Ff-Gracilis + SSG
3	Burn wounds thorax	SSG
4	Open wound after fracture of the femur	Free Rectus Abdominis + SSG
5	Open wound after rupture Achilles' tendon	SSG
6	Open wound after fracture ankle + OSM	SSG
7	Chronic open wound under foot	SSG
8	Deglovement thumb	SSG

SSG, split skin graft; Ff, Free flap; OSM, osteosynthesis material.

No differences were observed in age or nutritional status as expressed by Body Mass Index (BMI) between the different groups. BMI values of all patients were >20, and none of the patients suffered from weight loss >10% total body weight in the period of 6 months before inclusion in the study protocol. All burn wounds were <5% of total body surface, and all patients were metabolically stable. There were no differences in

donor site surface area comparing the placebo group (mean ± SEM)*:* 83.6 ± 17.9 cm^2 and arginine-treated group 67.5 ± 13.7 cm^2. During the study we experienced that most of the patients had difficulties drinking the amino acid solution because of the bad taste. Nausea and diarrhea occurred in two patients in the supplementation group during drinking of the solution on the first or second postoperative day. Vomiting in one patient, on the first post-operative day, but overall this patient drank 78% of the solution. Two patients in the supplementation group discontinued the study before the end because of this reason. These patients refused to participate in the follow up procedures (donation of blood and tissue samples) due to logistics reasons. In the placebo group similar tolerance was observed, two patients suffered from nausea. One patient did vomit on the second day postoperative, but overall intake was 87%. However, in the rest of the study subjects, all amino acid drinks were completely finished. No further adverse events were noticed. No clinical signs of infection of the donor sites were observed. In addition there were no quantitative bacterial cultures of the donor site >10^3 CFU observed in either group (data not shown).

Plasma amino acids

In the supplementation group a higher wound fluid arginine level was observed (Figure 5.1B). Significant higher citrulline levels were observed in wound fluid compared to plasma in both groups, indicating NO-production. However, no additional effect of arginine supplementation was observed on wound fluid citrulline concentrations showed no significant differences *between* groups (Figure 5.2B). Data showed no significant differences in wound fluid ornithine concentrations comparing both groups. Although, results show a tendency towards higher wound fluid ornithine levels in the supplementation group (Figure 5.3B).

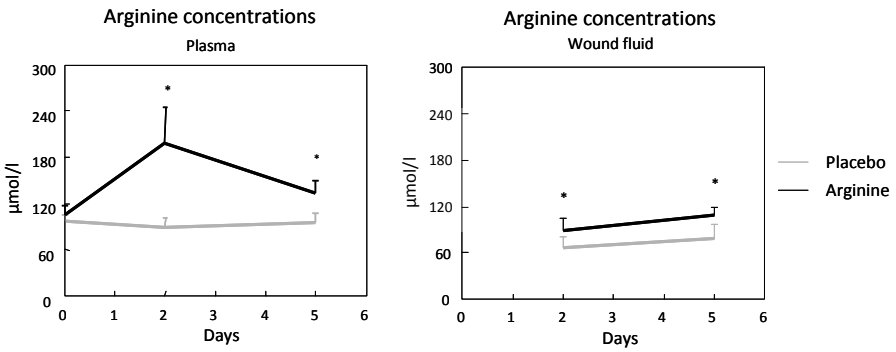

Figure 5.1 A and B: plasma and wound fluid arginine concentrations (µmol/l): placebo vs. suppletion. Data are expressed as means ± SEM, *p<0.05.

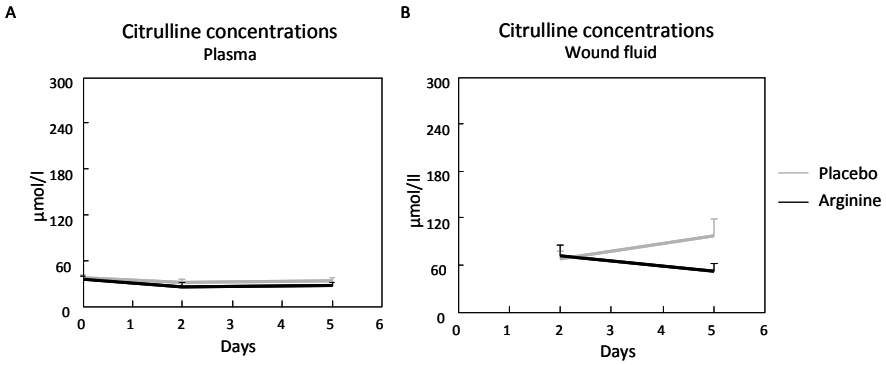

Figure 5.2 A and B plasma and wound fluid Citrulline concentrations (µmol/l); placebo vs. suppletion. Data are expressed as means ± SEM, *p<0.05.

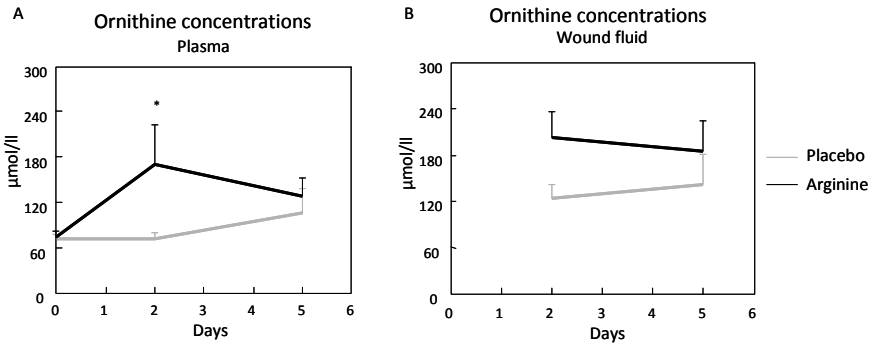

Figure 5.3 A and B: plasma and wound fluid ornithine concentrations (µmol/l); placebo vs. supplementation. Data are expressed as means ± SEM, *p<0.05.

Plasma NOx and wound fluid NOx

Data showed no differences in plasma NOx between the groups and no differences were observed in wound fluid NOx between both groups (Figure 5.4A, B).

Plasma and wound arginase-1

No significant differences were observed in plasma or wound fluid arginase levels between the control and supplementation group (Figure 5.5A, B).

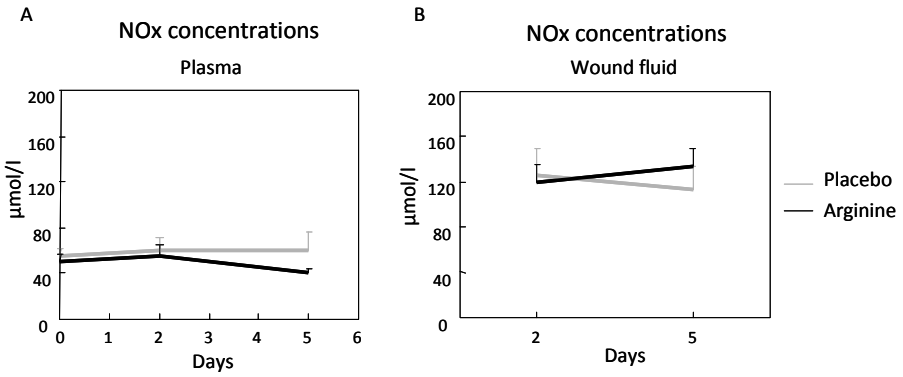

Figure 5.4 A and B: plasma and wound fluid NOx (sum of nitrate and nitrite) concentrations (µmol/l): placebo vs. supplementation. Data are expressed as means ± SEM, *p<0.05.

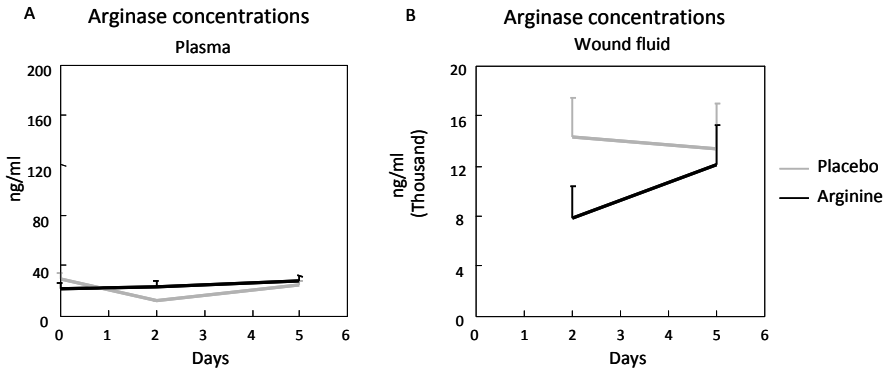

Figure 5.5 A and B: plasma and wound fluid arginase concentrations (ng/ml); placebo vs. supplementation. Data are expressed as means ± SEM, *p<0.05.

Evaluation of wound healing

PMN-count, angiogenesis and reepithelialization

Quantification of neutrophils, angiogenesis and reepithelialization, by blind histological investigation (kappa=0.81; 0.72; 0.56), showed no differences comparing both groups (Figures. 5.6, 5.7, and 5.8).

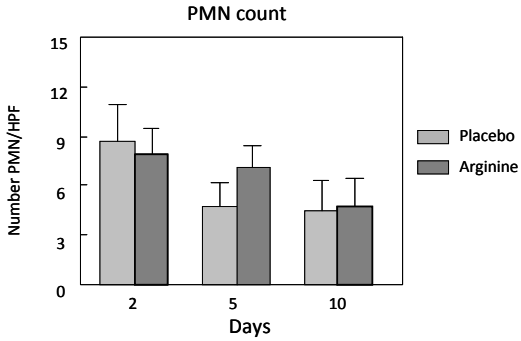

Figure 5.6 Polymorphonuclear neutrophils; number of PMN/HPF.
Data are expressed as means ± SEM.

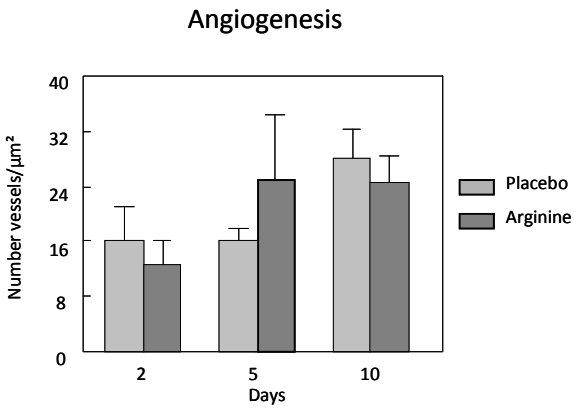

Figure 5.7 Angiogenesis; number of vessels/µm.
Data are expressed as means ± SEM.

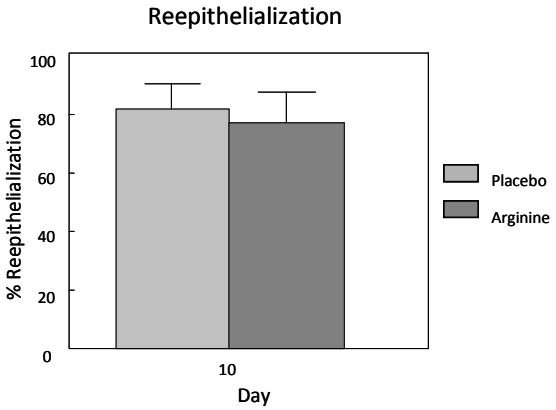

Figure 5.8 Reepithelialization; expressed as percentage of the total wound area. Data are expressed as means ± SEM.

Discussion

This pilot study was initiated to investigate the effects of oral arginine supplementation, as a single nutrient, on wound healing of clinical relevant surgical wounds in patients. Our study showed that *oral* arginine supplementation does not enhance reepithelialization of skin graft donor sites in relatively healthy patients. In addition, no effects were observed on arginine conversion in the wound environment comparing arginine-group to control group.

Oral arginine supplementation increased plasma arginine and ornithine levels, as well as local wound arginine and ornithine levels, the latter however not significant. This finding is comparable to prior studies[11,13] and can be explained by the fact that a large portion of L-arginine is passing through the gastrointestinal tract to the liver and is catabolized by arginase to ornithine and urea. The observed elevated wound fluid ornithine, could be a reflexion of these elevated plasma levels. However, previous studies show up regulation of arginase in wounded tissue (macrophages, fibroblasts etc), needed to generate substrates for collagen formation and cell proliferation[37-42]. This is confirmed by our findings as arginase-1 in wound fluid was much higher than plasma values. Although, arginase-1 is the predominant isoform in the liver and arginase-2 is found predominantly in extrahepatic tissues, with regard to wound healing, most studies show consistent increased expression of arginase-1 upon wounding[40-43]. Few show co-expression of arginase-1 and -2[39,44]. But the role of arginase-2 is not clear. Comparing our results to other human studies is difficult as no specific arginase-1 or 2 antibodies were used in their stainings[45,46]. Some show

increased expression of arginase in relation to impaired healing (chronic wounds and diabetic ulcers) and pathological skin conditions[47]. Only Corraliza et al., did measure arginase-2 in synovial fluid of arthritis patients using RT-PCR, and showed up regulation. They suggested that this up regulation deregulates the healing process in arthritis[48]. In the face of these previous results, our working hypothesis was that arginase-1 is the main pathway during human wound healing. In addition, no arginase-2 antibody for ELISA was available at the time of our experiments. Therefore, we chose to measure arginase-1 expression in plasma and wound fluid.

In the control group as well as in the supplemented group we observed NOS and arginase activity in the wound environment, as citrulline, NOx and ornithine levels were higher in wound fluid compared to plasma, this is in line with a well established experimental wound model[12,49]. However, our results show no additional effect in the conversion of arginine in the wound environment after supplementation. Although, not easy to compare to other studies as they not always evaluate plasma and wound fluid, our results are comparable[7,13,50]. In human studies, plasma arginine and ornithine are elevated after arginine supplementation[4,5]. The question rises why elevated arginine levels in wound fluid do not lead to increased NOS/arginase activity? Our wound model is an acute wound model in relatively healthy patients. As there was no clinical or histological difference in reepithelialization compared to controls, it is likely that NOS and arginase expression was maximally normal. In addition, enzyme-activity depends on substrate availability, and both our study groups showed no signs of arginine depletion in plasma or wound fluid.

PMN's are the first cells to arrive at the wound site; they degrade cell debris and extracellular matrix (ECM), decontaminate bacteria and produce cytokines to attract the more important macrophages[51]. In vitro studies showed that human neutrophils generate NO[52,53] and express NOS[54]. NOS peaks within 24-72 hours after wounding, inflammatory cells being the predominant source[12]. Different studies show beneficial effect of arginine supplementation on immune state[8,55] and decreased infection rate is observed upon arginine supplementation[17,18]. Moreover, Canturk et al. related an increased number and function of PMN's to increased in wound tensile strength, upon arginine supplementation[56]. Therefore, we evaluated the PMN-influx and quantitative bacterial analysis of the wound fluid. Furthermore, PMN's also regulate angiogenesis[57,58]. In vitro studies already showed the importance of NO in angiogenesis; iNOS-derived NO induces VEGF, the most potent known angiogenic protein[20-22]. In addition, Noiri et al. observed that inhibition of NO synthesis blunted VEGF-stimulated cell migration[59]. Most et al., showed that iNOS or eNOS (endothelial nitric oxide synthetase) knockout mice exhibited impaired VEGF expression and delayed wound healing[60]. Our results show no differences in PMN influx, infection or angiogenesis upon arginine supplementation. This could be explained by the fact that we studied acute wounds, in patients with a clinical good condition and clinically normal wound healing. Factors influencing enzyme expression, like diabetes were excluded from our study. Therefore, it is likely to assume that eNOS expression was

maximally normal. In addition, enzyme-activity depends on substrate availability, and both our study groups showed no signs of arginine depletion. Another possibility might be that specific immunomodulating effects of arginine only concern lymphocytes[4,5,37,61].

No differences in reepithelialization upon arginine supplementation were observed. Reepithelialization is NO-mediated as shown by several studies; proliferating keratinocytes of wound margins strongly express iNOS[51,62]. Inhibition of iNOS or eNOS impair epithelial proliferation [23,6,64]. In addition, there is a correlation between the availability of NO and keratinocytes proliferation[65]. Arginine supplementation in rats improved reepithelialization[16]. In the only human study evaluating arginine on reepithelialization of small experimental wounds in volunteers, no effect was observed[5]. The exact mechanism of action remains unclear. The proposed mechanisms of action of arginine supplementation on wound healing based on prior research are: 1) stimulation of adrenal and pituitary secretion of hormones, 2) arginine as biologic precursor for NO / arginase and 3) arginine as pharmaconutriënt. With regard to stimulation of adrenal and pituitary secretion of hormones there is controversy in literature. Some studies show increase of insulin-like-growth factor and growth hormone upon arginine supplementation in humans, while others refute this[66-68]. Arginine is the biologic precursor for NO / arginase, limited arginine availability is known to regulate iNOS and arginase activity. In our study no arginine depletion was observed in plasma or wound fluid, which might explain why there was no effect on reepithelialization. If there might be a depletion in the microenvironment which we could not observe, endogenous production of NO might be sufficient during normal wound healing. We did not measure hydroxyproline (OHP), so we cannot draw conclusions concerning collagen deposition, but with regard to comparable studies we might assume a same increasing effect. What is the reason for a potential effect on collagen deposition and not on reepithelialization? The role of arginine in collagen metabolism became clear after the finding that arginine becomes semi-essential after surgery[6]. Later on, several animal and human studies showed arginine supplementation improved OHP and wound breaking strength and that collagen synthesis is NO-mediated[69-72]. Others, suggested collagen-formation is more depending on arginine as precursor for OHP, which is an actual substrate for collagen building. The process of re-epithelialization is also closely controlled by growth factors, this process consists of multiple integrated networks of cell-matrix-cytokine interactions; it is possible that NO is only important in the early migration of keratinocytes, as Noiri et al. suggested[59]. And thereafter other factors like VEGF mediate the process[51,73]. Another explanation for the effect of arginine on collagen production might be the effect of ornithine. If supplemented as a single nutrient it also stimulates wound healing. Shi et al. showed that this effect was independent of NO and suggested that the available proline pool determines the rate of collagen synthesis[74]. In our study, we observed increased arginine as well as ornithine levels in plasma and wound fluid. It is possible that these amino acids are responsible for the difference in collagen-

formation vs. reepithelialization. The mechanisms of wound healing are complex, and dependent on temporally and spatially restricted NO expression. Therefore more research has to be done. Arginine as pharmaconutriënt: as arginine improves wound healing in animals, is a potent immunomodulator in humans and the role of NO is obvious in the different aspects of wound healing, we hypothesized that arginine might have the same influence on reepithelialization. However, we have to conclude from our results that arginine as a pharmaconutrient has no effect on reepithelialization in clinically relevant, but normal healing wounds. A restriction of this study may be the route of administration. Drinking the solution was sometimes difficult, causing nausea and vomiting. Two patients quit the study because of bad taste of the solution and were not included for analysis, the others completed the study and drunk the whole solution. Although this issue is important for future studies, it does not explain the negative results of our study. As this is a pilot study with small study groups, this could result in lack of statistical power. Most likely our wound model, which mainly examines reepithelialization, has restrictions in studying the effect of pharmaconutrients. Although epithelialization is important in wound healing and especially in burn wound treatment, a potential beneficial effect of arginine might be expected more in other types of wounds. Finally, our patients were relatively healthy; which might have contributed to the small differences found. It is likely that arginine supplementation in relatively healthy patients has not the same effect as compared to some animal species. The effect of arginine supplementation might be expected more in less healthy patients with an arginine depleted state like malnutrition, sepsis or cardiovascular disease. In conclusion, our results demonstrate that oral arginine as a pharmaconutrient has no effect on reepithelialization in clinically relevant, but normal healing wounds. Studies addressing our specific short comings might be initiated in the future.

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Chapter 6

Intravenous arginine and human skin graft donor sites healing; a randomized controlled trial

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Abstract

Background & Aims

Studies evaluating the effect of arginine supplementation in human wound healing are inhomogeneous and show conflicting results. This study aims to clarify the role of arginine supplementation in the healing of human skin graft donor sites.

Methods

35 subjects undergoing skin autografting were randomly assigned to receive intravenous arginine (n=16) or placebo (n=19) for 5 days in a dose of 30 g of arginine or an isovolumetric amount of placebo (25,2 g of alanine). Wound healing was evaluated at the donor sites by objectifying angiogenesis, reepithelialization and neutrophil influx. Plasma amino acid concentrations were measured to evaluate our intervention.

Results

The two groups were comparable in age, morbidity and nutritional, metabolic and inflammatory state. Plasma arginine and alanine levels increased significantly upon supplementation in the two groups, respectively. No differences were found between the arginine supplementation group and the placebo group in the studied parameters. Placebo vs. arginine; mean ± SD: *neutrophil influx on day 2*: 6.67±3.0 vs. 6.57±3.3, p=0.66; *angiogenesis on day 10:* 8.0±2.8 vs. 8.9±3.1; *reepithelialization in % on day 10*: 81±8.5 vs. 85±7.1.

Conclusion

Intravenous arginine supplementation does not improve angiogenesis, reepithelialization or neutrophil influx in healing of human skin graft donor sites.

Introduction

The effect of arginine supplementation on wound healing has been extensively studied in animals and has shown beneficial effects. A dietary lack of arginine leads to impaired wound healing in rodents[1,2], whereas arginine supplementation leads to increased wound collagen synthesis, wound breaking strength and reepithelialization[3-7]. While human studies are relatively scarce and show methodological flaws, data led to the production commercially available oral nutritional supplements, enriched with arginine, to increase wound healing. Until today however, the effect of arginine supplementation on the clinical outcome of wound healing in man remains to be clarified. In general, the effect of nutrition on wound healing, is well established[8]. In contrast, the role of immunonutrition and its specific ingredients to enhance wound healing is less established[9]. Human studies concerning the use of arginine as immunonutrition show conflicting results. While some studies show no beneficial effect of arginine supplementation on clinical or biochemical parameters in injured and post surgical patients[10-12], others indicate improved healing of diabetic ulcers[13], enhanced immune function, reduced postoperative infections, reduced postoperative wound complications and length of hospital stay in post surgical patients[14-22]. Experimental studies evaluating specific parameters of wound healing and arginine supplementation, such as collagen deposition and epithelialization, closest to our study objective, indicate enhanced wound healing[23,24]. Furthermore, most other studies have focused on patients in depleted state, suffering from malignancy, diabetes, pressure ulcers and severe trauma. These studies are often limited in size or express wound healing in terms of infection. Moreover, studies addressing the effect of arginine supplementation in wound healing often use combinations of arginine and other immunonutrients, which makes the interpretation of the single effect of arginine supplementation impossible[25-32]. Skin grafts are frequently used in reconstructive and burn wound surgery. Knowledge of possible means to enhance wound healing is particularly relevant in the treatment of burn wounds, as regrafting of a donorsite is often required[33]. In addition, a donor site is a very homogenous wound healing model. Therefore, the effect of oral arginine supplementation on healing of skin graft donor sites in relatively healthy humans was studied in a pilot study previously conducted at our department. No beneficial effects of arginine supplementation were found, on angiogenesis, reepithelialization and leukocyte influx as parameters of wound healing in patients undergoing skin autografting[34]. The beneficial effects, as suggested by previous literature prompted us to conduct a study that overcomes the possible shortcomings of our previous pilot study. In this double blinded, randomized controlled trial we included a larger study population and administrated arginine intravenously, in order to rule out any influence on study results due to intake problems. Wound healing was objectively evaluated as primary end point, using angiogenesis, reepithelialization and neutrophil influx as key parameters.

Materials and methods

Design and subjects

Between July 2006 and July 2009 a randomized double blind, placebo-controlled study was performed at our department. The Medical Ethical Committee of Maastricht University Hospital approved the study protocol, and informed consent was obtained for each subject. The sample size for this study was calculated based on mean total healing time of donor sites of 10 days, with 80% confidence and accepting an α of 5%. In order to detect a increased healing of 15%, 25 subjects per group would have to be included. All subjects received skin autografting as part of reconstructive surgery. Body mass index and weight loss within six months prior to surgery was measured to determine clinical nutritional status according to the Espen guidelines. Exclusion criteria were age younger than 16 or older than 75 years, kidney or liver failure, pregnancy, use of steroids, immune deficiency diseases, and diabetes mellitus. Before operation subjects were randomly assigned to arginine (n=16) or placebo treatment (n=19), by an independent clinical pharmacist, using numbered envelopes. A block randomization was chosen to equally divide subjects in both groups. Based on literature at the initiation of the study, the highest tolerable amount of arginine was administered. Subjects received intravenous supplementation of arginine or placebo (Bufa, Uitgeest, The Netherlands) during five days, starting during surgery, in order to evaluate the different processes during the initial phases of wound healing (inflammatory and proliferative phase). Intravenous supplementation consisted of either a daily dose of 30 grams of arginine, dissolved in 1000 ml 0.9% NaCl and adjusted to pH 7,2 using 10% HCL (net nitrogen intake 45,7 mmol/l) or a placebo treatment consisting of a daily dose of 25,2 grams of alanine, dissolved in 1000 ml 0,9% NaCl (net nitrogen intake 44 mmol/l). To be able to perform a double blinded study the infusions were made isovolumetric and isonitrogenous. Although it was not possible to make them isocaloric, the arginine infusion accounted for 120 kcal and the placebo infusion for 100.8 kcal. However, in a metabolic stress situation, these small differences are often neglected. Patients received a daily infusion of 1000 ml in two doses of 500 ml equally divided over the day. Oral food intake was allowed as desired, in order to maintain the clinical applicability of arginine as a potential immunonutrient treatment.

Wound model

All clinical wound procedures were performed at the Department of Plastic Surgery, University Hospital Maastricht, The Netherlands. Under general anesthesia and aseptic conditions, split skin grafts were obtained using an electric dermatome (Aesculaap®) with a thickness of 0.3 mm. The donor sites were used to evaluate wound healing. Wound fluid was collected from these donor sites by covering it with a layer of Gordasoft® (homemade sterilized polyester fabric), followed by a polyvinyl alcohol

sponge (Coldex®, Taureon, Rijswijk, The Netherlands) and a transparent dressing (Tegaderm®, 3M Nederland B.V., Zoeterwoude, The Netherlands) on top as previously described.
The surfaces of all donor sites were measured. Twenty-four hours before wound fluid collection the wound dressing was changed by removing the transparent dressing and the sponge, followed by reapplying a new sponge and transparent dressing. After twenty-four hours the sponge was removed, and stored on ice until further processing. Using this protocol a twenty-four hour wound fluid sample was used each time for analysis. From each patient wound fluid samples were taken using this method on day 2, 5 and 10. From the central part of the donor site 3-mm punch biopsies were taken. Before the excision of the biopsies, lidocaine was locally injected. Subsequently a venous blood sample was drawn from a major vein in the cubital fossa.

Sample processing and analysis

Heparinized blood was centrifuged at 4°C for 10 minutes at 4000 rpm within one hour after sampling. After centrifugation, 500 µl of plasma was deproteinized using 20 mg dry sulphosalicylic acid (SSA), vortexed and frozen in liquid nitrogen. Samples were stored at -80 degrees Celsius until analysis. Wound fluid was obtained by centrifuging the sponges for 10 min at 4°C (11.000 rpm). After centrifugation, 500 µl of wound fluid was treated similar to the plasma. The recovery of fluid from the sponges was validated and found to be constant.

Evaluation of wound healing

In order to study the different phases of wound healing, donor site biopsies were obtained at identical time points: post-operative day 2, 5 and 10. These time points were chosen to reflect the inflammatory phase at day 2, the proliferative phase at day 5 and reepithelialization on day 10. Biopsies were fixed in 4% formaldehyde, processed by routine histological procedures and embedded in paraffin. Four µm sections were subsequently obtained from each paraffin block.

Immunohistochemical assessment of neutrophil-influx and angiogenesis

Sections were initially stained with haematoxylin and eosin. Polymorphonuclear neutrophils (PMN's) were counted per high power field (HPF) at a magnification of 40 in wound biopsies sections of day 2, 5 and 10 post-surgery. We objectified angiogenesis by micro vessel density (MVD), as described by Hillen et al and Baeten et al. in wound biopsies of day 2, 5 and 10 post-surgery[35,36]. Immunolabeling of mouse-anti-human alpha smooth muscle actin (ASMA; Dako Laboratories, Carpinteria, CA, USA) and mouse-anti-human CD31 (Dako Laboratories Carpinteria, CA, USA) were used to stain vessels in the sections. Two separate blinded investigators performed the assessments using a microscope .

Reepithelialization

Standardized photographs of the donor sites with measuring scales were taken on days 5 and 10 (Canon EOS 300 with macro lens, 1:1). Two blinded independent observers measured the surface of reepithelialized skin by planimetry using the morphometric program ImageJ (W. Bailer, Hagenberg, Austria). The area with reepithelialized skin was expressed as a percentage of the total wound area (central part). A quantitative bacterial analysis was performed of wound fluid samples, expressed as colony forming units (CFU).

Biochemical analysis

Amino acids measurement in plasma

Arginine, citrulline and ornithine concentrations were measured in plasma of all patients. Levels were determined using a fully automated HPLC system as described previously[37].

Statistical Analysis

Data were expressed as means ± SEM. Amino acid concentrations were expressed in µmol/l. Repeated-measures ANOVA was used to compare both groups. A 95% confidence interval was used to determine significance. The statistical package for social sciences (SPSS) program was used for statistical analysis.

Results

Forty patients complied with the selection criteria and were included in the study. Twenty subjects were randomly allocated to receive placebo and 20 subject to receive arginine. In the placebo group one patient discontinued the study and in the arginine supplementation group four patients discontinued after one day. All subjects discontinued for the same reason: after dislocation of the intravenous needle refusing a new needle only for study purposes. Therefore thirty-five patients were analyzed. Both formulas were well tolerated and both groups consumed 100%. Table 6.1 shows patient characteristics. Age ranged from 17 to 74 years (mean ± SEM: 44±17). No statistical difference was observed between the two groups in age. Both groups had a comparable nutritional and metabolic status as expressed by BMI (all patients were >19,7), total protein and albumin. With regard to weight loss 6 months prior to the study, as an indication of nutritional depletion, 3 patients suffered from >10% loss of their weight. Two patients were in the arginine group (11% in 1,5 month; 25% in 9 months) and one in the placebo group (12% in 3 months). No adverse effects like vomiting, diarrhea were observed. Infection parameters as expressed by CRP were low

and not statistically different in both groups. Details on performed operations can be found in Table 6.2.

Table 6.1 Patient characteristics.

Parameter	Control group *Mean (SEM)*	Arginine group *Mean (SEM)*
Male/Female	9:10[A]	11:5[A]
Age (years)	44,6 (4)	42,3 (4,2)
Weight (kg)	82,9 (5,6)	77,4 (4)
BMI (kg/cm^2)	27,2 (1,7)	25,1 (1,0)
CRP (mg/l)	13,3 (3,3)	10,8 (3,1)
Albumine (g/l)	30,7 (1,1)	33,4 (1,5)
Total plasma protein (g/l)	62,4 (2,6)	68,8 (2,9)

[A] absolute numbers

Table 6.2 Operations.

	Control group *(n=19)*	Arginine group *(n=16)*
Reason for operation		
Burns	2	3
Trauma	9	7
Malignancy	4	1
Other	4	5
Type of operation		
SSG*	13	7
Muscle transposition + SSG	1	1
Free muscle transfer + SSG	5	8

* SSG = split skin graft

Plasma amino acids

Plasma arginine and ornithine levels increased significantly on post-operative days 2 and 5, indicating adequate supplementation (Figure 6.1 and 6.2). The increased plasma ornithine levels can be explained as a conversion of the supplemented arginine by liver arginase. We observed no changes in plasma citrulline levels (Figure 6.3), nor plasma sum of amino acids (sumAA) (Figure 6.4). The later indicates that both groups have comparable catabolic state. In Figure 6.5 levels of plasma alanine are shown, increased levels were observed on day 2-5 in the control group, which normalized at day 10, suggesting adequate intake.

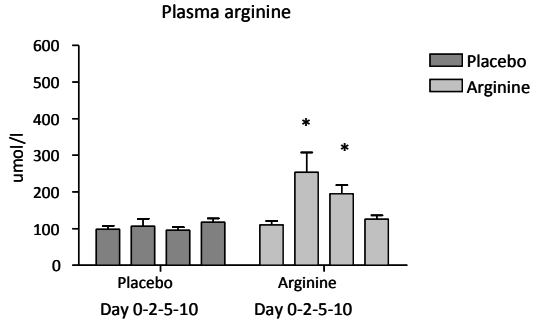

Figure 6.1 Plasma arginine concentrations (µmol/l): placebo vs. suppletion. Data are expressed as means ± SEM, *p<0.05. Adequate rise of plasma arginine is observed upon supplementation.

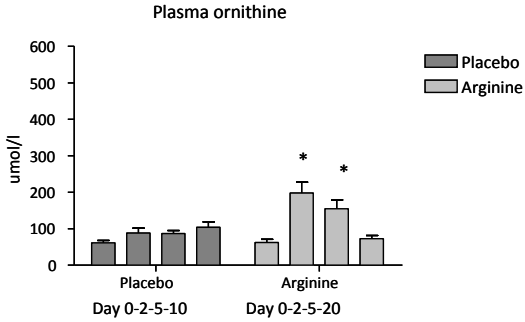

Figure 6.2 Plasma ornithine concentrations (µmol/l); placebo vs. suppletion.
Data are expressed as means ± SEM, *p<0.05. Upon arginine supplementation, significantly increased plasma ornithine levels are observed, indicating arginase activity.

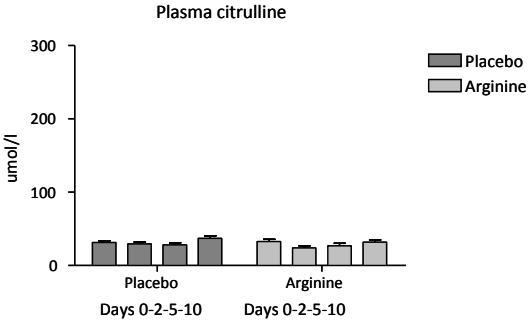

Figure 6.3 Plasma citrulline concentrations (µmol/l); placebo vs. suppletion.
Data are expressed as means ± SEM, *p<0.05. Arginine supplementation does not affect the NOS-pathway.

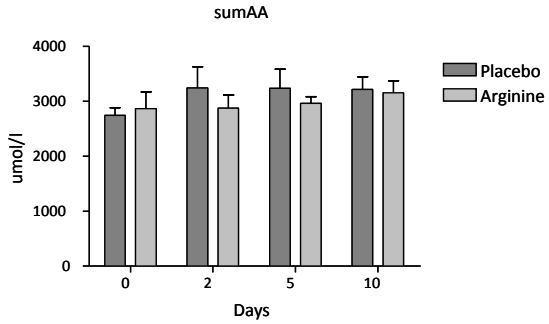

Figure 6.4 Plasma sumAA concentrations (µmol/l); placebo vs. suppletion.
Data are expressed as means ± SEM, *p<0.05. We observed no changes in plasma sum of amino acids, suggesting that both groups have comparable catabolic state.

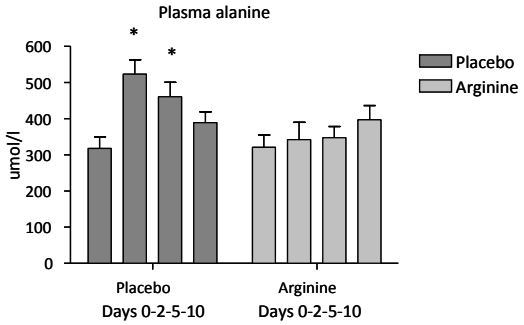

Figure 6.5 Plasma alanine concentrations (µmol/l); placebo vs. suppletion.
Data are expressed as means ± SEM, *p<0.05. The placebo group showed adequate rise of plasma alanine levels.

Evaluation of wound healing

PMN-count, angiogenesis and reepithelialization

No statistical differences were found between the two groups in quantification of neutrophils, angiogenesis and reepithelialization (Figures 6.6, 6.7 and 6.8). Intra- and interobserver variability being respectively 0,98 and 0,73 for quantification of neutrophils and 0,97 and 0,83 for quantification of angiogenesis.
Wound neutrophils (Figure 6.6) decreased significantly in both groups between day 2 and day 10 (p=0,008 for both groups) and day 5 and day 10 (p=0,049 for control group and p=0,019 for arginine supplementation group). There was no significant difference in decrease on subsequent days between the two groups.

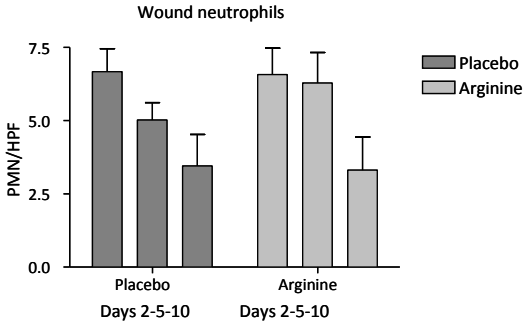

Figure 6.6 Polymorphonuclear neutrophils; number of PMN/HPF.
Data are expressed as means ± SEM. PMN's as a measure of immuunrespons were not affected by arginine supplementation.

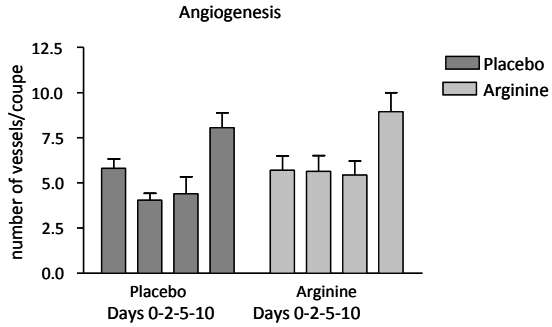

Figure 6.7 Angiogenesis; number of vessels/µm.
Data are expressed as means ± SEM. Although a tendency was seen toward increased neovascularization on day 10, no differences were observed between both intervention groups.

Angiogenesis (Figure 6.7) expressed as the number of vessels in wound tissue per high power field show significantly increased vessel count in both the control group and the arginine suppletion group between day 0, 2 and day 10 (respectively p=0,007 and p<0,001). No significant differences were found for the number of vessels on the subsequent days between both groups.
The amount of wound reepithelialization, expressed as percentage of the total wound surface (Figure 6.8), was similar for both groups on day 5 and day 10 post-surgery.

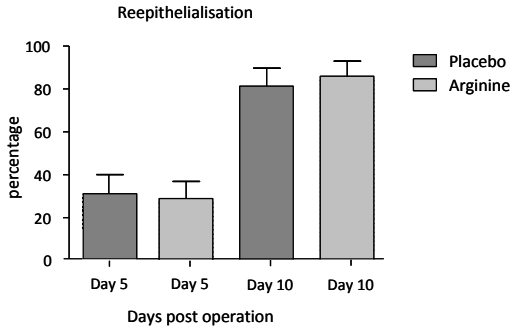

Figure 6.8 Reepithelialization; expressed as percentage of the total wound area. Data are expressed as means ± SEM. No differences were observed between both groups on post-wounding days 5 and 10.

Discussion

The aim of our study was to evaluate the effect of arginine on wound healing in relatively healthy humans with clinically significant wounds. To overcome possible compliance problems with oral intake, we used intravenous arginine administration. No differences were found between the two groups on evaluation of reepithelialization, angiogenesis and wound neutrophil influx as parameters of wound healing.

Plasma amino acids were studied in order to evaluate the effect of administration of arginine on blood arginine concentration. Both plasma arginine and ornithine increased significantly in the supplementation group on day 2 and day 5 and normalized by day 10. Increased ornithine levels are explained by the conversion of arginine to ornithine by the hepatic enzyme arginase. Despite the high activity of hepatic arginase, arginine levels raise significantly, showing an effective administration and hence biological availability. When arginine is metabolized by nitric oxide synthase (NOS), producing NO, citrulline is produced in equivalent amounts. Despite the coupled production of NO and citrulline, plasma citrulline cannot be used as marker of systemic NO-synthesis as it is converted back to arginine. This de novo arginine synthesis mainly takes place in the kidneys, but has also been described in endothelial cells and macrophages in vitro[38,39]. In the wound environment, however, citrulline and nitrite/nitrate levels are used as NO-synthesis markers. Rodents show a simultaneous increase of both NO and citrulline levels at the wound site in the first 48 hours after wounding[40,41]. Similar findings were observed in humans in our previous study[34]. However, no additional effect was observed after arginine supplementation. Present study reveals lack of differences in plasma citrulline between the two groups, which is

in line with the previous pilot study. These findings can either be explained by the conversion of systemic citrulline back to arginine or by an unchanged NO production despite the relatively high arginine supplementation. Similar findings were recently published by Lo *et al*, showing that a pharmacological dose of parenteral arginine did not cause augmentation of the NO production in sub-acute inflammation in peritonitic rats[42]. These findings might be explained by enzyme kinetics. Several factors influence the catalytic reaction, such as temperature, pH, the amount of enzyme, the amount of substrate and the presence of inhibitors. After arginine supplementation, serum levels rise above the Km value of NOS (2-20 μM)[43,44]. This indicates that the amount of substrate arginine is more than enough to activate NOS, and thus producing NO. Moreover, present study shows that elevated substrate levels do not increase arginine conversion. This might explain why arginine supplementation is not effective.

Plasma alanine was significantly increased in the placebo group on days 2 and 5 and normalized by day 10. No differences were found for total plasma amino acids levels in both groups on all days, indicating comparable metabolic states between the study groups.

Neutrophil influx was assessed to evaluate the essential inflammatory phase of wound healing. Neutrophils degrade cell debris and extracellular matrix, decontaminate bacteria and produce cytokines, but also generate NO[45-47]. As mentioned before, NO is being considered as the central signal molecule in several important processes in wound healing. Several studies have indicated an increased immune response on the administration of arginine[6,14,15,22,24,26]. Others found a decreased infection rate upon arginine supplementation[18,25]. Our results showed no differences in wound neutrophil influx for both groups on all days, suggesting similar wound inflammatory reaction in both groups. In line with this finding, no clinical wound infections were observed in both study groups (data not shown).

Angiogenesis was assessed as a second parameter of clinical wound healing. The process of angiogenesis is mediated by NO, through the induction of VEGF. In vitro studies show that human neutrophils generate NO[46,47]. Experimental studies have hypothesized that increased arginine levels stimulate local NO production by wound neutrophils, which would enhance NO-dependant processes, such as angiogenesis. Assessment of angiogenesis in our patients, however, shows no differences upon arginine supplementation on all post-operative days.

Reepithelialization, the third and true clinical parameter of wound healing in our study, has been shown to be enhanced by arginine supplementation in several animal studies, but not in human studies[24,48,49]. Results of the present study, however, showed no enhanced reepithelialization in the supplementation group on day 10 of wound healing.

Our results differ from and contradict the findings of other authors. Different explanations can be proposed for our findings. First, most previous human studies evaluate derivatives of wound healing as primary endpoints, looking at wound infection, wound complication or length of hospital stay, whereas we look at more

specific parameters of wound healing. Interpretation of relations between these different endpoints is difficult. Second, the included subjects in our study population might cause the discrepancy in results. We included relatively healthy subjects, while others included patient groups with extensive (co) morbidities and therefore depleted metabolic states[13-22]. In addition, different types of wounds were studied. Some used small experimental wounds and others chronic wounds, which are not as homogenous as the donor sites of our subjects[24,50]. A third possible explanation might be the differences in species, which makes it difficult to extrapolate the results of experimental studies to human studies. And finally, clinical trials evaluating the effect of arginine, often use a combination of immunonutrients, which makes it impossible to draw any conclusions on the effects of arginine solely[25-32].

Although the number of subjects of our study is not sufficient to draw firm conclusions, results suggest that intravenous arginine is not beneficial for host immune response, angiogenesis or reepithelialization of skin graft donor sites in relatively healthy humans. Despite a significantly increased plasma arginine level after supplementation and the lack of side effects. We therefore hypothesize that arginine supplementation might only affect wound healing when there is a depletion of arginine, systemically or locally. Future studies are needed to assess the effect of arginine and other amino acid supplementation in different clinical wounds and/or patient groups.

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Summary and conclusions

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The skin is the most important barrier against hostile attacks from outside our body. However, it also helps us to interact with our environment. When damaged, a complex cascade of repair mechanisms is initiated to restore its function. This is a well-balanced process, influenced by many mediators. Although research has led to new therapeutical interventions in wound healing, delayed wound healing still is a major burden for patients and society. One of the mediators for adequate skin regeneration is nutrition. The main objective of this thesis was to investigate the isolated role of the immunonutrient arginine in the healing of surgical and chronic wounds in men. We hypothesized that arginine supplementation improved human wound healing.

In the **first chapter ,** a review of literature on the current knowledge of the role of arginine in wound healing is presented. Arginine was found to be an important nutrient for skin repair in animal studies and its supplementation significantly improved wound healing. Early results from few studies in men on experimental wounds were promising. As a consequence, arginine supplementation quickly found its way into clinical practice. Clinical studies followed, however they mainly used commercially available nutritional supplements containing, besides arginine, other nutrients such as nucleotides and ω-3 fatty acids. The use of a mixture of nutrients and often the lack of a good control group, supplied with isonitrogenous and/or isocaloric amounts, make it difficult to conclude whether treatments should be attributed to the specific pharmaconutrients or simply to the addition of amino acids and/or calories in general. Furthermore, the parameters used to evaluate wound healing were indirect e.g. infection, or pain free removal of the dressing.

The first step in our thesis was to investigate wether human skin uses arginine for its regeneration, therefore in **chapter 2** the skin's transorgan metabolic routing of arginine was investigated, an opportunity given to us by free vascularized tissue surgery. Previously, arginine conversion was assessed indirectly. However, the studies presented here used labelled amino acids (isotopes) to study true metabolism. Patients undergoing reconstructive breast surgery received a skin flap named deep inferior epigastric artery (DIEP) flap. During this surgery an arginine tracer ($^{15}N_2$-arginine) was infused and blood was sampled from the DIEP-flap. Significant uptake of $^{15}N_2$-Arginine ($p<0.05$) was observed. Although we failed to prove the enzymatic conversion to ^{15}N citrulline or $^{15}N_2$ urea, arteriovenous concentration differences of citrulline and ornithine show that the skin produces both amino acids. Since both amino acids are not protein bound they must be generated endogenously within the skin flap by NOS and ASE-activity respectively. Although tracer data clearly show arginine uptake by the skin, net arginine release was observed. In line with the net release of most other amino acids this finding can be ascribed to net protein breakdown in these post-absorptive patients. The sole net uptake of the amino acid glutamate was remarkable.

Next step was to look at the expression of arginine-metabolic enzymes and arginine-metabolites, which were expected to be elevated in surgical wounds compared to normal skin. In **chapter 3** data are presented of our immunohistological study, in which several biopsies were taken from wounds during a period of ten days post-wounding. We observed that macrophages, polymorphonuclear neutrophils (PMN's), fibroblasts, keratinocytes and endothelial cells showed expression of inducible nitric oxide synthetase (iNOS) compared to no/sparse expression in normal skin. Furthermore, we observed constitutive expression of endothelial nitric oxide synthetase (eNOS) in vessels. After injury an increased number of vessels was observed expressing iNOS. Moreover, both arginase isoforms, arginase-1 (ASE-1) and arginase-2 (ASE2), were expressed during wound healing. ASE1 was uniquely expressed by neutrophils post-wounding, while ASE-2 staining was observed in endothelial cells, keratinocytes, fibroblasts, macrophages and PMN's. In addition to immunohistological evaluation of wounds, levels of arginine metabolites were measured. Increased levels of wound fluid citrulline, nitrite/nitrate (NOx) and ornithine were measured compared to plasma, indicating arginine conversion by NOS and ASE. Levels of ASE1 were significantly higher in wound fluid compared to plasma, while levels of ASE2 were higher in plasma. On the basis of our observations we conclude arginine consumption during healing of surgical wounds in men.

In **chapter 4** the expression of arginine metabolites/metabolic enzymes in chronic wounds were compared to acute wounds. Because infection complicates the course of wound healing, we related the presence of infection to altered arginine metabolism. Some significant differences were observed. First, infected chronic wounds showed significantly elevated wound fluid levels of citrulline and ornithine in comparison to acute wounds. As these amino acids are not released from protein degradation, this suggests elevated activity of the enzymes NOS and ASE. This is supported by the finding of significantly elevated wound fluid ASE1 in these wounds. In contrast decreased levels of NOx were observed in wound fluid of infected chronic wounds, indicating decreased nitric oxide synthesis. Furthermore, patients with infected chronic wounds showed decreased plasma arginine levels compared to patients with acute or non-infected chronic wounds.

In **chapter 5** we describe the results of a double blinded randomized clinical pilot study investigating the effect of oral arginine supplementation, as a single nutrient, on the healing of skin autograft donorsites. Wound healing was evaluated by measuring PMN-influx, neovascularisation and reepithelialization. In both groups equal NOS and ASE-activity was observed in the wound environment. Patients receiving arginine, showed no improvement of wound healing.

In **chapter 6** we tried to overcome possible shortcomings of the previously conducted pilot study, by enlarging the study population and supplying arginine intravenously. The same wound model and wound healing parameters were studied. Both groups were comparable in age, nutritional/metabolic and inflammatory state. We observed an adequate raise of plasma arginine after supplementation. However, no beneficial

effect was observed on PMN-influx, infection, angiogenesis or reepithelialization upon arginine supplementation.

Final conclusions and future perspectives

The expression of NOS-isotypes and arginine metabolites in surgical wounds presented in this thesis supports previous experimental studies indicating the need for arginine in a NO-mediated repair of injured skin. In addition, we observed arginase expression during healing. Previous studies showed elevation of ASE1 in animal wound healing and overexpression of ASE1 in pathological skin conditions in men. However, our results suggest a more prominent role of ASE2 in normal healing in men. It is not unimaginable that different wound types have different needs at different time points. Our study on chronic wounds supports this hypothesis by showing elevated arginine conversion compared to acute wounds. Furthermore, a NO-depleted state was observed in the infected chronic wounds, possibly responsible for defective defense barrier, as NO is used by several inflammatory cells. This deficient NO production might be caused by bacterial arginine consumption. Another explanation might be a process called substrate competition known from in vitro experiments. A disturbance of the delicate balance of NOS and ASE expression, favoring one of both metabolic routes, might affect the availability of arginine. Remarkably, patients with infected chronic wounds had decreased levels of plasma arginine. Lowering of systemic arginine levels is also seen in patients subjected to trauma, burns or surgery and not beneficial for the immune system. As patients had no different systemic inflammatory response, no differences in nutritional state or co-morbidity compared to non-infected chronic wounds, we attributed this finding to a increased metabolic demand caused by the infection present in the wound.

We also conclude that arginine supplementation does not improve wound healing in acute surgical wounds. This is in sharp contrast to experimental studies. We used the donorsite of skin autografting as our model. The advantage of this model is that it is homogeneous and easy to access. Disadvantage can be that it can only be used to study reepithelialization. Although a clinically relevant model, a donor site heals quickly under normal circumstances with few complications. Therefore, differences might be difficult to observe. It could be that arginine supplementation is more effective in other wounds types, like chronic wounds with a NO-depleted state. Sequential activation of the different arginine metabolic pathways is observed in animal models. This might indicate a time course requirement for different arginine metabolites. Moreover, topical application of arginine might be explored, as topical NO-donors have shown beneficial effects on experimental wound healing. Although, arginine plasma levels rose adequately after supplementation, we also observed high levels of plasma ornithine. Bypassing the catabolization of arginine by the gastrointestinal tract should be considered. Citrulline is a natural precursor for L-arginine, it is converted to arginine in many tissues. Therefore it bypasses

metabolism in the liver and is not a substrate or inducer of arginase. Others already showed increased levels of plasma arginine accompanied by induction of bioactive NO upon citrulline supplementation.

Finally, from our study using stable isotopes we might conclude that arginine consumption by skin is not a substrate driven process. In the future, we should consider exploring the possibilities of manipulating the expression of arginine-metabolizing enzymes in wounds. Although the animal experimental data are compelling, there is need for more experimental and clinical trials in order to better define the role of arginine in the care of patients.

Samenvatting en conclusie

Samenvatting en conclusie

De huid is onze belangrijkste barrière tegen schadelijke invloeden van buitenaf. Tevens helpt ze ons in de communicatie met onze omgeving. Wanneer de huid beschadigd is, wordt een complexe cascade van herstelmechanismen in gang gezet om haar functie te herstellen. Dit goed uitgebalanceerde proces wordt beïnvloed door vele mediatoren. Hoewel onderzoek met betrekking tot wondgenezing heeft geleid tot vele nieuwe therapeutische interventies, blijft vertraagde wondgenezing een zware last voor de patiënt en de samenleving. Voeding is van essentieel belang voor een adequate wondgenezing, waarbij aminozuren de bouwstenen zijn. Aminozuren zijn de bouwstenen van onze voeding. Dit proefschrift beschrijft de rol van het aminozuur arginine als immunonutrient, bij de genezing van chirurgische en chronische wonden bij mensen. Onze hypothese is dat arginine suppletie humane wondgenezing zal verbeteren.

In het **eerste hoofdstuk** wordt een literatuuroverzicht van de huidige kennis over de rol van arginine in de wondgenezing gepresenteerd. Uit dierproeven blijkt dat arginine een belangrijke voedingsstof is voor het herstel van de huid. Als immunonutrient verbetert arginine de wondgenezing aanzienlijk. Ook de resultaten van enkele vroege studies met experimentele humane wonden waren veelbelovend. Naar aanleiding van deze resultaten vond suppletie van arginine al snel haar weg naar de kliniek. De daaropvolgende klinische studies vertoonden echter nogal wat beperkingen. Zo bevatten de toegediende voedingssupplementen naast arginine ook andere bestanddelen zoals nucleotiden en omega-3-vetzuren. Vaak ontbrak een goede controlegroep, gesuppleerd met isonitrogene en/of isocalorische hoeveelheden aminozuren. Moest het effect van deze behandelingen toegeschreven worden aan de specifieke immunonutriënten of aan de toevoeging van aminozuren en/of calorieën in het algemeen? Bovendien werden indirecte parameters gebruikt om de wondgenezing te evalueren zoals infectie of het pijnvrij verwijderen van verbandmiddelen. Deze beperkingen maakten het moeilijk conclusies te trekken uit deze studies.

De eerste stap voor dit proefschrift was het toetsen van de hypothese dat de menselijke huid arginine gebruikt. **Hoofdstuk 2** beschrijft te resultaten van de transorgane meting van arginine metabolisme in de huid. Dit werd ons mogelijk gemaakt doordat plastische chirurgie gebruik maakt van zogenaamde vrij gevasculariseerde weefsel transplantatie, waarbij het huidmetabolisme relatief geïsoleerd bestudeerd kan worden. Tot nu toe werd arginine metabolisme indirect beoordeeld. Echter voor onze studie werden gelabelde aminozuren (isotopen) gebruikt. Patiënten die een borstreconstructie ondergingen middels een vrij gevasculariseerde huidlap, genaamd Deep Inferior Epigastric Artery flap (DIEP-lap) werden geïncludeerd. Tijdens deze operatie werd een arginine tracer ($^{15}N_2$-arginine) toegediend en tevens werd bloed afgenomen uit de ader en slagader van de DIEP-lap. Een significante opname van $^{15}N_2$-arginine ($p < 0,05$) werd waargenomen. Hoewel we de enzymatische omzetting naar ^{15}N citrulline of $^{15}N_2$ ureum niet konden aantonen,

kunnen we uit de arterioveneuze concentratieverschillen van citrulline en ornithine opmaken dat de huid deze aminozuren produceert. Omdat geen van beide aminozuren vrijkomt uit eiwitafbraak, moeten deze endogeen gegenereerd zijn in de huid door respectievelijk NOS en ASE-activiteit. Ondanks dat de tracer data duidelijk aantonen dat er arginine opname door de huid plaatsvindt, werd een netto arginine release waargenomen. In lijn met de netto release van de meeste andere aminozuren kan deze bevinding toegeschreven worden aan netto eiwitafbraak in deze katabole patiënten. Een opmerkelijk bevinding was dat het aminozuur glutamaat als enige een netto opname vertoonde.

De volgende stap was het bestuderen van de expressie van enzymen die betrokken zijn bij arginine metabolisme in zowel de normale huid als in de huid met chirurgische wonden. Hierbij werd een verhoogde expressie in chirurgische wonden ten opzichte van normale huid verwacht. **Hoofdstuk 3** beschrijft de resultaten van onze immunohistologische studie, waarbij biopten van wonden gedurende een periode van tien dagen na de operatie werden bestudeerd. We constateerden toegenomen expressie van induceerbaar stikstofoxide synthetase (iNOS) in macrofagen, polymorfonucleaire neutrofielen (PMN's), fibroblasten, keratinocyten en endotheelcellen in deze chirurgische wonden vergeleken met normale huid. Verder constateerden we een basale expressie van endotheliale stikstofoxidesynthetase (eNOS) in bloedvaten van de normale huid. In de wonden echter werd een verhoogd aantal bloedvaten waargenomen die tevens iNOS tot expressie brachten. Zowel de arginase isovormen, arginase-1 (ASE1) en arginase-2 (ASE2) bleken tot expressie te komen tijdens de wondgenezing. ASE1 komt alleen tot expressie in neutrofielen na verwonding, terwijl ASE2-aankleuring wordt waargenomen in endotheelcellen, keratinocyten, fibroblasten, macrofagen en PMN's.

In aanvulling op immunohistologische evaluatie van wonden, werden de waarden van arginine metabolieten gemeten in wondvocht en plasma. Hieruit kan men indirect arginine metabolisme afleiden. Er werden verhoogde waarden citrulline, nitriet / nitraat (NOx) en ornithine gemeten in wondvocht ten opzichte van plasma. Waarden van ASE1 waren significant hoger in wondvocht in vergelijking met plasma, terwijl de waarden van ASE2 hoger waren in het plasma. Deze waarnemingen suggereren arginine verbruik tijdens de humane wondgenezing. Om een potentiële verklaring te vinden voor de verstoorde heling van chronische wonden wordt in **hoofdstuk 4** de expressie van arginine metabolieten en metaboliserende enzymen in chronische wonden vergeleken met chirurgische wonden. Aangezien infectie het beloop van wondgenezing negatief beïnvloed, werd tevens de relatie tussen de aanwezigheid van een infectie en arginine-omzetting bestudeerd. Derhalve hebben we de chronische wonden in twee subgroepen verdeeld, te weten de niet- geïnfecteerde en de geïnfecteerde. Enkele significante verschillen werden waargenomen. Ten eerste, geïnfecteerde chronische wonden laten significant verhoogde citrulline en ornithine waarden zien in wondvocht ten opzichte van chirurgische wonden. Aangezien deze aminozuren niet uit eiwitafbraak vrijkomen, suggereert deze verhoging een

toegenomen omzetting van arginine door de enzymen NOS en ASE. Deze suggestie wordt ondersteund door de bevinding dat deze geïnfecteerde wonden significant hogere ASE1 concentratie bevatten. Er werden echter verlaagde waarden van NOx waargenomen in wondvocht van geïnfecteerde chronische wonden, hetgeen kan duiden op een verminderde aanmaak of verhoogd verbruik van stikstofmonoxide. Een NO deficiëntie kan nadelig zijn voor de afweer reactie. In tegenstelling tot de andere twee subgroepen werd er in de groep patiënten met geïnfecteerde chronische wonden verlaagde plasma argininespiegels geconstateerd. Aangezien er geen verschil was in systemische metabole of inflammatoire status tussen de subgroepen patiënten, wijzen deze bevindingen op het feit dat toegenomen lokaal verbruik van arginine in chronisch geïnfecteerde wonden leidt tot een verlaging van systemische arginine spiegels.
In **hoofdstuk 5** worden de resultaten van een pilotstudie beschreven. De opzet van de studie was een dubbelblinde gerandomiseerde klinische studie die het effect van orale arginine-suppletie, als mononutriënt, op de genezing van donorsites (van huidtransplantaten) bestudeert. De wondgenezing werd geëvalueerd door het meten van PMN-instroom, neovascularisatie en reëpithelialisatie. In zowel de controle als gesuppleerde groep werd een gelijke NOS en ASE-activiteit waargenomen in het wondmilieu. Patiënten in de arginine-groep, toonden geen verbetering van de wondgenezing.
In **hoofdstuk 6** werd gepoogd de mogelijke beperkingen van de eerder uitgevoerde pilotstudie te verbeteren door de studiepopulatie te vergroten en arginine intraveneus toe te dienen. Hetzelfde wondmodel en dezelfde parameters werden bestudeerd. Beide groepen waren vergelijkbaar in leeftijd, nutritiële, metabole en inflammatoire status. Een adequate stijging van het plasma arginine werd na suppletie waargenomen. Een gunstig effect op de wondgenezing bleef echter uit.

Definitieve conclusies en toekomstperspectieven

De expressie van NOS-isovormen en argininemetabolieten in chirurgische wonden zoals beschreven in dit proefschrift ondersteunt de eerdere experimentele studies waaruit blijkt dat het herstel van beschadigde huid gereguleerd wordt door NO. Daarnaast werd arginase expressie waargenomen tijdens de wondgenezing. Eerdere studies toonden een verhoging van ASE1 in dierlijke wondgenezing en een overexpressie van ASE1 in pathologische huidaandoeningen bij mensen aan. Echter, onze resultaten tonen een meer prominente rol van ASE2 in normale humane wondgenezing. Het is niet ondenkbaar dat verschillende typen wonden verschillende behoeften aan voedingsstoffen hebben op verschillende tijdstippen. Onze studie over chronische wonden ondersteunt deze hypothese door een verhoogde arginine conversie aan te tonen in vergelijking met chirurgische wonden. Verder werd een NO-deficiëntie waargenomen in de geïnfecteerde chronische wonden, mogelijk verantwoordelijk voor de gebrekkige afweer, aangezien NO wordt gebruikt door verschillende inflammatoire cellen. Een tekort aan NO zou ook veroorzaakt kunnen

worden door bacteriële arginine consumptie. Een andere verklaring kan gezocht worden in een proces genaamd “substraat competitie” en bekend van in-vitro experimenten. Dit is een verstoring van het evenwicht in NOS en ASE expressie, waarbij één van beide metabole routes de overhand krijgt. Deze verstoring kan dan invloed hebben op de beschikbaarheid van arginine. Verder was het opmerkelijk dat patiënten met geïnfecteerde chronische wonden verlaagde plasma arginine spiegels vertoonden. Verlaging van de systemische arginine spiegels wordt ook gezien bij patiënten die zijn blootgesteld aan een trauma, brandwonden of operatie en deze verlaging is niet bevorderlijk voor het immuunsysteem. Onze verschillende subgroepen patiënten lieten geen verschil zien in systemische inflammatoire response, voedingstoestand of comorbiditeit. Derhalve zijn wij van mening dat een verlaagde plasma arginine spiegel toegeschreven kan worden aan een verhoogde lokale metabole behoefte ten gevolge van een infectie.

Een andere belangrijke conclusie van dit proefschrift is dat arginine-suppletie de wondgenezing in acute chirurgische (schaaf)wonden niet verbetert. Dit staat in schril contrast met experimentele studies. Als wondmodel gebruikten we de donorsite van de huidtransplantaten. Het voordeel van dit model is dat het homogeen is en gemakkelijk toegankelijk. Nadeel kan zijn dat deze voornamelijk gebruikt kan worden om reëpithelialisatie te bestuderen. Hoewel het een klinisch relevant wondmodel is, geneest een donorsite snel onder normale omstandigheden, hetgeen een reden kan zijn dat verschillen moeilijk waar te nemen zijn. De bevindingen van deze studie sluiten niet uit dat arginine suppletie potentieel meer effect heeft op andere typen wonden.

Mogelijk toekomstig onderzoek kan zich richten op de behoefte aan arginine in de verschillende fasen van wondgenezing. In diermodellen is reeds een sequentiële activering van de verschillende metabole routes van arginine waargenomen. Dit kan duiden op een tijdsafhankelijke behoefte aan verschillende arginine metabolieten. Daarnaast zou lokale applicatie van arginine kunnen worden onderzocht, aangezien het aanbrengen van een NO-donor op wonden in de vorm van een crème een gunstig effect heeft op experimentele wondgenezing. Citrulline suppletie kan eveneens onderzocht worden. Hoewel arginine plasmaspiegels adequaat stegen na suppletie, werden ook hoge plasmaspiegels van ornithine waargenomen. De omzetting van arginine door het gastro-intestinale kanaal dient hiervoor verantwoordelijk te worden gehouden. Citrulline is een natuurlijke precursor voor L-arginine en het wordt omgezet in arginine in vele weefsels. Citrulline omzeilt het metabolisme in de lever en is geen substraat of inductor van arginase. Verhoogde argininespiegels in plasma en tegelijkertijd inductie van het bioactieve NO zijn reeds aangetoond na citrulline suppletie. Tot slot kunnen we van onze studie met stabiele isotopen concluderen dat arginine consumptie door de huid niet een substraatgedreven proces is. Het lijkt daarom meer logisch te focussen op manipulatie van expressie van arginine-metaboliserende enzymen in wonden in plaats van substraat te suppleren. Ondanks de positieve experimentele data, is er meer behoefte aan klinische studies om de rol van arginine beter te definiëren in de zorg voor patiënten.

Dankwoord

Dankwoord

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Curriculum Vitae

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Iris Barbara Johanna Gertruda Debats werd geboren op 19 september 1975. Na het afronden van het gymnasium aan de Stedelijke Scholengemeenschap te Maastricht, ging zij geneeskunde studeren. Eerst aan het Limburgs Universitair Centrum, te Diepenbeek (B) en later aan de Universiteit van Maastricht. In augustus 2001 behaalde zij haar arts examen.
Hierna heeft zij gewerkt in Medisch Centrum De Heel, te Zaandam, als ANIOS algemene heelkunde. Om vervolgens in april te starten bij de vakgroep Plastische Chirurgie, te Maastricht. Na een aantal maanden als ANIOS gewerkt te hebben werd zij fulltime onderzoeker voor een periode van twee en een half jaar (opleider Prof. dr. W. Boeckx). De vooropleiding chirurgie volgde zij in het Orbis Medisch Centrum, te Sittard (opleider Dr. A.G.M. Hoofwijk). Haar vervolgopleiding startte zij in januari 2007 in het academisch ziekenhuis te Maastricht. De perifere stage werd gevolgd in het Atrium Medisch Centrum (Th. Van der Kar) en na het behalen van het EBOPRAS examen, rondde zij haar vervolg opleiding succesvol af (opleider Prof. dr. Van der Hulst).
Van 1 juli 2010 tot eind 2011 zal zij werkzaam zijn als fellow in de Hand en Pols chirurgie op de afdelingen Plastische, Reconstructieve en Handchirurgie verbonden aan de volgende ziekenhuizen; in Rotterdam het Erasmus Medisch Centrum (Prof. dr. S.E.R. Hovius); in Den Haag het Haga Ziekenhuis (R. Koch); in Amsterdam het Amsterdams Medisch Centrum (Dr. S. Strackeé)en de Vrije Universiteit ziekenhuis (Prof. M. Ritt); in regio Utrecht het Diakonessenhuis (Dr. T. Moojen/R. Feitz).

Curriculum Vitae

The author of this thesis, Iris Barbara Johanna Gertruda Debats, was born in Bilzen, Belgium, on September 19th 1975. After graduating the Gymnasium at the Stedelijke Scholengemeenschap Maastricht in 1993, she started her medical education at Limburgs Univeritair Centrum, Diepenbeek (B). After two years she switched to Maastricht University and finished her medical education in august 2001.
After medical university, she decided to leave the Southern part of the Netherlands and worked as a resident surgery at Medical Centre De Heel, Zaandam. This was only for a short period, after half a year she came back to Maastricht, to the department of Plastic, Reconstructive and Handsurgery, University Medical Centre. For 3 years she committed to this department as a resident and fulltime researcher on arginine and wound healing. From January 2005 to 2007 she worked as a resident at the department general surgery in Sittard (Head dr. A.G.M. Hoofwijk). In January 2007 she started her residency Plastic, Reconstructive and Handsurgery at Maastricht University Hospital and Atrium Medical Centre in Heerlen (Head Prof. dr. R. van der Hulst, Drs. Th. Van de Kar). Her residency was completed after she successfully took the EBOPRAS exam.

From 1st of July 2010 until the end 2011 she will be working as a clinical fellow, specializing in hand and wrist surgery, at the departments of Plastic, Reconstructive and Hand Surgery, at the Erasmus Medical Centre Rotterdam (Prof. dr. S.E.R. Hovius); Haga Hospital in the Hague (R. Koch); Amsterdam Medical Centre (Dr. S. Strackeé) and VU Medical Centre in Amsterdam (Prof. dr. M. Ritt); Diakonessenhuis in Utrecht/Zeist (Dr. T. Moojen/R.Feitz).